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IMMUNOLOGICAL INVESTIGATIONS
INTO
SYNAPTIC PLASTICITY

By

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A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy.
The Brain and Behaviour Research Group
The Open University, Milton Keynes.

Submitted on June 3rd, 1991.

**Here is the number on the door,
Memory,**

You have the key.

(From *Rhapsody on a Windy Night* by T.S. Eliot)

Extraordinary the tricks that memory plays!

(Vladimir in *Waiting for Godot* by Samuel Beckett)

(Changing attitudes to a PhD 1987-1991!)

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LIST OF ABBREVIATIONS.

STM	Short-term memory
LTM	Long-term memory
ITM	Intermediate term memory
CS	Conditioned stimulus
US/UCS	Unconditioned stimulus
GTP	Guanosine triphosphate
AMP	adenosine triphosphate
cAMP	cyclicAMP
PKC	Protein kinase C
PKA	Protein kinase A
EPSP	Excitatory postsynaptic potential
LTP	Long-term potentiation
NMDA	<i>N</i> -methyl-D-aspartate
PCP	Phencyclidine
APV	D,L-2amino-5-phosphonovalerate
MAP2	Microtubule-associated protein 2
PSI	Protein synthesis inhibitor
PSD	Postsynaptic density
HVc	Rostral hyperstriatum ventrale/Higher vocal centre
RA	Round nucleus of archistriatum

2-DG	2-deoxyglucose
MeA	Methyl anthranilate
IMHV	Intermediate medial hyperstriatum ventrale
PA	Paleostriatum augmentatum
LPO	Lobus parolfactorius
kDa	Kilodalton
SPM	Synaptic plasma membrane
RNA	ribonucleic acid
mRNA	Messenger RNA
2-D-gal	2-deoxygalactose
M	Methylantranilate-trained
W	Water-trained
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
EEG	Electroencephalograph
ELISA	Enzyme-linked immunosorbent assay
OG	Octyl glucoside
INT	p-iodonitrotetrazolium violet
TBS	Tris-buffered saline
IgG	Immunoglobulin G
PBS	Phosphate-buffered saline
TEMED	Tetramethylethylenediamine

MW	Molecular weight
N-CAM	Neural cell adhesion molecule
SMF	Synaptic membrane fraction
PIRS	Pre-immune rabbit serum
PMSF	Phenylmethanesulphonylfluoride

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ABSTRACT

Antibody technology was applied to the study of synaptic plasticity resulting from passive avoidance training in the chick and long-term potentiation in the rat. These studies fell into three categories: 1) the disruption of memory for a one-trial passive avoidance task by intracranial injection of an anti-postsynaptic density antiserum 2) mapping time- and locus-specific changes in the chick brain using antisera to the cytoskeletal proteins α -tubulin and microtubule-associated protein 2 following passive avoidance training and 3) using antibodies to synaptically-enriched antigens to map time- and locus-specific changes in hippocampal subfields following long-term potentiation in the rat.

In the first series of experiments an antiserum (R14) was raised against protein from chick forebrain postsynaptic densities (PSDs). The antiserum was characterised and was found to recognise six distinct antigens as determined by Western Blots. These antigens were found to have a primarily (but not exclusively) synaptic location. Intracranial injections of IgG isolated from R14 resulted in amnesia for a one-trial passive avoidance paradigm in the chick when administered 60min pre-training (but not 30min or 15min pre-training or 10min post-training), in chicks tested 24hrs (but not 1hr or 3hrs) post-training.

In the second set of experiments monoclonal antibodies were used to examine changes in levels of the cytoskeletal proteins α -tubulin and microtubule-associated protein 2 (MAP2) in specific forebrain loci following passive avoidance training in the chick. Of the regions examined, elevations in the titre of anti- α -tubulin were found in the left Intermediate Hyperstriatum Ventrale (IMHV) 1hr, 6hrs and 24hrs following passive training, in the left Lobus Parolfactorius (LPO) 1hr following training and in the right LPO 6hrs and 24hrs following training. A hemispherically-asymmetrical change was found in the titre of anti-MAP2 which was interpreted as possibly reflecting a decrease in the amount of the

antigen in the left IMHV 24hrs following training. No training-related changes were detected, using either antibody, in a third forebrain region, the Paleostriatum Augmentatum (PA).

During the characterisation of antiserum R14 it was found that only one antigen (with an apparent molecular weight of 230kDa) is conserved between the chick and rat brain. The antigen is enriched in synaptic fractions isolated from the rat hippocampus and was used, as well as a PSD-specific monoclonal antibody, 411B, to examine possible changes in hippocampal subfields CA1, CA3 and the dentate area taken at several time-points following tetanisation of the right perforant path. 24hrs following tetanisation (but not at earlier time-points), the titre of R14 was elevated in the dentate area ipsilateral to tetanisation and in both the ipsi- and contralateral CA1. The titre of 411B was increased specifically in the target, dentate area and only at 8hrs following tetanisation, an increase which was abolished in the presence of the protein synthesis inhibitor, anisomycin.

These results are discussed in the context of current models of synaptic plasticity.

CHAPTER 1: INTRODUCTION

1i) THE BIOLOGY OF LEARNING AND MEMORY.

It is the capacity for learning and memory and the ability to retrieve information when appropriate, which allow all animals to survive by adapting to events in their environment. It endows the organism with the means to modify behaviour with respect to past experience within its lifespan and, in more complex animals, to represent cause and effect relations within the world. The underlying mechanisms of learning and memory are of enormous interest to the neurobiologist.

Historically, the biology of memory has been the subject of much speculation and it has only been in the latter half of this century that techniques have been developed to allow rigorous scientific investigation of the phenomena at anything other than the behavioural level. Our knowledge of the underlying biochemical and physiological mechanisms of memory is still sparse despite an enormous amount of progress in the last two decades.

Much of the early information about the parameters of learning and memory was gleaned from studies of humans performing in laboratory tasks and from studies of amnesias following head trauma or brain damage. However, most of the progress in understanding the biological mechanisms has been achieved through the use of animal models, in which an organism is trained on a task which reliably results in learning and memory as expressed by some behavioural measure. These types of studies fall into two main categories; the use of specific interventive techniques which disrupt memory formation, maintenance and/or retrieval, and the assaying of brain tissue for biological changes following (and in some cases during) training.

Subdivisions of memory.

Studies on humans and other organisms have clearly demonstrated that different types of memory exist. Notably, in many types of memory, there appears to be a clearly defined division between a short-term memory (STM) phase lasting from the onset of the remembered experience to minutes after it, and long-term memory (LTM) which lasts from minutes to hours and is probably permanent.

The evidence for this distinction comes from several sources. Humans who have endured head trauma (including electroconvulsive shock) typically cannot remember the trauma or events which occurred in the minutes immediately preceding it (retrograde or pre-morbid amnesia), suggesting that the forgotten events were in a labile short-term store which was disrupted by the incident. Longer-term memories remain unaffected, indicating that by the time of the trauma they have already entered a more durable store.

Some patients who have received brain damage, particularly to the hippocampal formation and limbic structures, are unable to retain new memories for longer than minutes (anterograde or post-morbid amnesia). These rapidly decay and are lost, while events preceding the damage remain intact, suggesting that the affected brain areas are necessary either for the storage of long-term memories or for the passage of information from STM to LTM.

More evidence for the STM/LTM distinction results from interventive techniques in animals. If an animal is trained on a simple task and is injected with drugs of known biochemical action within specific time windows relative to training, the animal can be rendered amnesic in one of two ways. An intracranial injection of protein synthesis inhibitor (e.g. anisomycin or emetine), will disrupt LTM while STM remains unaffected.

Alternatively, an injection of a drug such as ouabain which disrupts ion channel function by inhibiting sodium/potassium ATPase activity, results in the loss of both early and late memory. The fact that STM cannot be inhibited without also affecting LTM strongly supports the hypothesis that STM and LTM are sequential processes, i.e. that a memory must pass through STM before being coded in LTM. These type of experiments also suggest that the mechanisms which lead to the formation of long-term memory are set in motion at the onset of learning since there are specific time windows, usually around the time of training, in which inhibitors of LTM must be administered in order to render an animal amnesic (e.g. Patterson et al., 1986; 1988). There is also evidence for an intermediate-term memory (ITM) lasting from minutes to around an hour which is uniquely susceptible to particular drugs. The biological substrates of STM, ITM and LTM and the use of animal models will be discussed in more detail in later sections.

Before examining the nature of biological representations, it is necessary to briefly define types of learning. These fall into three main categories. Associative learning refers to situations where an animal learns connections between related events in its environment (classical conditioning) or between its behaviour and events in the environment (operant conditioning). Non-associative learning refers to the phenomenon whereby an animal strengthens reflexes in response to a single, usually strong, stimulus (sensitisation) or decreases a reflex in response to a repeated, often weak, stimulus (habituation). Incidental learning describes situations where an animal appears to learn about events in the absence of an identified connection between stimuli.

How is a memory represented in the brain of an animal which undergoes a learning experience? During embryonic development, the nervous system is established and organised in a similar way to other organs. Unlike other organs however, the functional units of the brain, neurons, do not divide further once they have been laid down. It is

changes in the pattern and strength of connections between neurons which are believed to subserve memory formation and representation. Some of the general rules for such changes in synaptic efficacy were expounded in Donald Hebb's germinal work "The Organisation of Behavior" (Hebb, 1949). Hebb presented a "neuropsychological theory" in which the short-term representation of memory is coded by electrical reverberation in neuronal circuits and long-term memory by enduring morphological changes within these circuits.

Although at present the techniques are not available to study memory-related changes in single neuronal circuits or cell assemblies, it is probably fair to say that most researchers in the field assume that a Hebbian-type mechanism is involved in the establishment and retention of memory. The majority of memory-related changes which have been found in the biological properties of synapses are compatible with such a model.

1ii) THE USE OF ANIMAL MODELS.

An enormous number of different animal species, from slugs and snails to rats and primates, have been used in attempts to elucidate the biological substrates of learning and memory. An extensive review of every paradigm in every organism is obviously beyond the scope of this thesis. This section will concentrate on learning and memory paradigms in three major areas in which the biological mechanisms of synaptic plasticity have been extensively (though by no means completely) described. These will include invertebrate preparations (including the marine molluscs *Aplysia* and *Hermisenda* and the insect, *Drosophila*), the use of mammals to delineate types of memory, the phenomenon of long-term potentiation and, finally, avian learning and memory especially in the precocial chick (including imprinting but with particular emphasis on passive avoidance training).

1iii) Learning and memory in invertebrates.

One approach which has yielded much information about the biochemical substrates of learning and memory is the use of simple invertebrate preparations, in particular the marine molluscs *Aplysia californicus* and *Hermissenda crassicornis* and the fruitfly *Drosophila melanogaster*. The advantage of using such organisms is the relative simplicity of the invertebrate nervous system which typically comprises of hundreds or thousands of more or less identifiable, large neurons. Because of its enormous contribution to the understanding of the cellular mechanisms of simple memory formation, research using *Aplysia* will be reviewed in some detail whereas relevant findings from studies of *Hermissenda* and *Drosophila* will be considered at less length.

Aplysia.

The work of Kandel and colleagues has revealed much about the biochemistry underlying simple learning involving the gill withdrawal reflex in *Aplysia*. The neurons which subserve the reflex have been identified and the animal has been described as being "built like an old Philco radio, with simple circuits and large, easily identifiable components" (Quinn, 1984). If the gill and mantle shelf of *Aplysia* is stimulated by a harmless stimulus, such as a light touch or a jet of water, the gill is withdrawn. The withdrawal reflex readily habituates if the stimulus is presented repeatedly. Alternatively, presentation of a noxious stimulus, usually a mild electric shock to the tail, will result in a generalised increase in the magnitude of all responses - including the gill withdrawal (sensitization) (e.g. Kandel, 1976).

The animal is also capable of learning in an approximation to classical conditioning. In this paradigm, the conditioned stimulus (CS), a weak tactile stimulus to the siphon, is presented less than a second before the unconditioned stimulus (UCS), a shock to the tail. After a number of paired presentations the CS alone will elicit a gill withdrawal of the same magnitude as that observed in response to the UCS alone. The behavioural parameters which define habituation and distinguish it from simple fatigue in the neural pathways were laid down by Thompson and Spencer (1966). For example changing the stimulus which is presented to a habituated reflex will restore the magnitude of the response. Thus habituation can be regarded as learning in that the behaviour manifested by the memory is stimulus-specific and retained over time (repeated habituation of the antennae withdrawal response in the land snail *Helix aspersia* can result in habituation lasting for at least months (Scholey and Ray, 1986)). Habituation of the gill withdrawal reflex in *Aplysia* follows these rules and certain cellular correlates of this simple form of learning have been identified. Notably, Castelluci and Kandel (1974) reported a decrease in the number of neurotransmitter quanta released by habituated cells. However, most of the biochemical data concerning non-associative phenomena come from studies of sensitization and the classical conditioning paradigm.

The neurons which subserve the gill withdrawal reflex consist of sensory neurons, motor neurons, facilitatory neurons, and interneurons. The connections between two types of neurons are crucial to sensitization of the gill withdrawal reflex. Sensory neurons, which innervate the skin, synapse on motor neurons which underpin the gill withdrawal reflex. These same sensory neurons receive presynaptic inputs from facilitatory neurons that innervate the tail (Kandel, 1976; Hawkins et al., 1983).

It is the presynaptic input received by sensory neurons from the facilitatory neurons which is believed to be responsible for the biochemical changes which underlie

sensitization of the gill withdrawal reflex via a mechanism of presynaptic facilitation. Transmitter released from the facilitatory neuron activates a second messenger cascade in the presynaptic terminal of the sensory neuron according to the following scheme (e.g. Goelet et al., 1986).

Neurotransmitter binds to presynaptic receptors which causes activation of the G-protein G_s which binds GTP resulting in the stimulation of the catalytic subunit of adenylate cyclase. The conversion of adenylate cyclase to cyclic AMP (cAMP) switches on cAMP-dependent protein kinase (Schwartz et al., 1983). This kinase phosphorylates an outward K^+ potassium channel which, in its non-phosphorylated form, is responsible for repolarization of the cell following an action potential. The delayed repolarization that follows phosphorylation results in a broadening of the action potential, an increased calcium influx and an increase in the amount of neurotransmitter released from the sensory neuron onto its motor neuron. This means that the motor neuron is more likely to fire and the magnitude of the response to be increased (Bardarletti and Siegelbaum, 1988).

In the classical conditioning paradigm used in *Aplysia*, Kandel proposes that the UCS results in an identical cascade to that described above for sensitization. The CS is represented by spike activity in the same sensory neuron which is facilitated by the UCS. Spike activity results in an influx of calcium into the neuron and, since the adenylate cyclase is sensitive to Ca^{2+} /calmodulin, is also capable of activating the catalytic subunit of this enzyme. Persistent modification of this molecule is hypothesised to represent the behavioural changes observed in the intact animal. (Hochner et al., 1986).

In the scheme proposed by Kandel the adenylate cyclase molecule represents the point at which the CS and the UCS converge. This model can be criticised on a number of points. Firstly the conditioning paradigm itself is unusual in that the conditioned stimulus itself

evokes a response - it is merely the magnitude of the response which changes after pairing with the unconditioned stimulus. In addition to this, Kandel's biochemical scheme does not adequately address the problem of why, at the behavioural level, the conditioned stimulus must predict the unconditioned stimulus. Secondly, the model suggests that the site of learning is located in the presynaptic cell, whereas many other studies, particularly in vertebrates indicate a postsynaptic or pre/post synaptic site of learning. The third point of criticism is that many researchers in the field reject Kandel's dogmatic reductionism, believing rather that memory is a property of cell assemblies rather than single molecules.

Hermisenda.

Another marine mollusc, *Hermisenda crassicornica*, has been used in studies of the underlying biology, particularly biophysiology, of learning and memory (especially in the work of Alkon and colleagues). When presented with light, the animal normally displays a positive phototaxis which is a result of lengthening of the animal's foot. Pairing presentation of light with rotation leads to suppression of this behaviour by shortening the foot (Lederhendler et al., 1986).

The neural pathways of the visual and vestibular pathways of *Hermisenda* survive surgery and much of the work on *Hermisenda* comes from biophysical measurements of isolated neural preparations following training or other interventions. The pathways converge in the primary sensory neurons of the eye, the photoreceptors. Measurements in both type A and particularly type B photoreceptors reveal changes in these cells' biophysical characteristics as a results of conditioning (Alkon, 1983; Crow, 1984).

Short-term changes in cellular properties of trained animals include increased spontaneous activity and excitability resulting from a more depolarised resting potential. Long-term correlates include an increased input resistance. Axotomised photoreceptors continue to exhibit light-evoked receptor potentials (Crow and Alkon, 1980) and trained animals' photoreceptors maintain the training-related alterations in cellular properties demonstrating that these changed properties are intrinsic to the cells and, once established, independent of sustained synaptic input. These alterations in membrane properties have been explained in terms of a persistent inactivation of a voltage-dependent K^+ conductance across the resting cell membrane (Alkon et al., 1982; 1987a) which, like *Aplysia*, appeared to result from phosphorylation involving the cAMP-dependent kinase cascade (Alkon et al., 1983). Experiments have also indicated central roles in mediating the K^+ channels for the Ca^{2+} /calmodulin-dependent protein kinase II (e.g. Sakakibara et al., 1985) and protein kinase C (Farley and Auerbach, 1986).

Although the specific parts played by the above kinases is unclear, the studies do demonstrate a clear role for Ca^{2+} -dependent phosphorylation cascades in the Hermissenda conditioning paradigm.

The specific mechanism proposed by Alkon is outlined below. The model requires an unusual temporal contiguity in that effective conditioning results from simultaneous cessation of the CS and US, (Farley, 1986). In the model both light (CS) and rotation (US) result in elevated surges of intracellular calcium which activate the Ca^{2+} /calmodulin-dependent protein kinase and/or the Ca^{2+} /phosphoinositol mediated activation of PKC. This results in phosphorylation of the outward rectifying K^+ channel increasing the excitability of the type B photoreceptors by an enhanced Ca^{2+} influx which can feed back into the mechanism. Repeated training then results in maintained phosphorylation and effective closure of the K^+ channel.

Retention is believed to be a result of the enduring closure of the K^+ channels. The closed channels mean that the repolarising K^+ channel is diminished so that subsequent presentation of light evokes an enhanced receptor potential. The B photoreceptors have inhibitory synapses on medial type A receptors which themselves excite the muscles subserving positive phototaxis (Lederhendler et al., 1986).

The activation of PKC could be mediated by the calcium surges described above or by a neuromodulatory transmitter in the US pathway. This latter possibility was tested by using a "conditioning analogue" in which the photoreceptors were presented with light and candidate neuromodulatory transmitters were substituted for the US (rotation) (Sakakibira et al., 1987; Crow and Forrester, 1986). Similar biophysical changes to those found following training occurred with serotonin but not with dopamine or octopamine. As with training, the changes depended on the temporal pairing of light with neurotransmitter. The most important cellular correlate of the conditioning analogue is an enhanced generator potential which by 24hrs persisted only in the group which received paired light and serotonin group. Although these results shed promising light on the possible mechanisms of memory formation in *Hermissenda*, no elevation in levels of serotonin have been found following activation of the US pathway by rotation. It is possible that serotonin may act in parallel with identified CS and US pathways to amplify conditioning-induced changes.

There also appears to be a role for protein synthesis in memory consolidation in *Hermissenda*. As with other preparations, the short-term cellular correlates of training are not blocked by anisomycin whereas the long-term enhancement of generator potential is anisomycin-sensitive (Alkon et al., 1987).

Drosophila.

Other invertebrates which have been used in the studies of learning and memory include the fruitfly, *Drosophila melanogaster*. In an associative learning paradigm the insect learns to associate shock with a distinctive odour. If one odour is paired with shock and another with reward the insect learns to make the appropriate response and this learning is retained for at least 5 hours (Quinn et al., 1979).

A number of *Drosophila* mutants have been isolated which are incapable of learning this task (e.g. Dudai et al., 1976) including the imaginatively named "Dunce", "Turnip", "Cabbage" and "Rutabaga" and the mutant "Amnesiac" which can learn the task normally but exhibits low retention after about an hour.

All the mutants have well-defined metabolic differences compared to normal, wild-type strains, for example Rutabaga has abnormally low levels of cAMP (Byers et al., 1981) whereas the Dunce strain has elevated cAMP levels (Livingstone and Temple, 1983). Metabolic cascades normally operate at optimal, rather than maximum levels and quantitative kinetic modelling has revealed that increasing cAMP to abnormally high levels results in lower efficiency of the PKA activation (Buxbaum and Dudai, 1989).

The temporal parameters of the behavioural deficits are compatible with those of Kandel's model in that they confirm a central role for the cAMP cascade in learning and short-term memory. However, one cannot rule out the possibility of deficits in the cAMP cascade system causing developmental perturbations (for example in neuronal wiring) which would result in the reported learning abnormalities.

The use of invertebrate preparations in the study of the neurobiology of learning and memory has revealed a great deal about the cellular components of the processes. Many of the cellular components described in invertebrates are highly phylogenically conserved and have been replicated in higher organisms. Vertebrate memory, however, is likely to include highly complex organisational properties which cannot be delineated through the use of invertebrates.

1iv) Learning and Memory in Mammals.

Many mammal species have been used in the study of learning and memory. While being less amenable to studies of the cellular concomitants of plasticity, mammals (rats and mice in particular) have been used extensively in the characterisation of memory systems (it has often been said that the white rat along with the psychology undergraduate are the favourite tools of the behavioural psychologist).

Operant conditioning tasks have been used in many studies of mammalian memory. These include the use of rats, cats, dogs and primates (e.g. Perin, 1943; Bitterman, 1975). Such studies have become somewhat unfashionable because operant learning requires many trials, may not reflect biological adaptiveness and there is a lack of generality across species (Seligman, 1970).

Both active and passive avoidance tasks in the rodent have been utilised in neurobiological studies of learning and memory. Such tasks often require the animal to learn to escape from an aversive stimulus such as footshock and are learned in only a few trials, allowing the relatively quick biological analysis of brain tissue. Passive avoidance paradigms have been used in rats (e.g. Morgan and Routtenberg, 1979; Nolan et al, 1987a; 1987b) and

mice (e.g. Izquierdo and McGaugh, 1987). Related tasks include those in which brightness discrimination is used so that the animal must enter a lighted compartment in order to avoid punishment (Wetzel et al., 1980; Popov et al., 1983).

Other approaches using mammals to study the mechanisms of plasticity include the use of non-learning interventions such as comparing animals reared in complex and impoverished environments. Rats which were exposed to many salient, bright and colourful stimuli during neonatal development had more synapses per neuron (Greenough et al., 1985) and increased electrophysiological activity as measured by population excitatory post-synaptic potential (EPSP) and granule cell population spike (Green and Greenough, 1986).

Such studies are difficult to interpret, however, since what is described as an "enriched" environment is probably much closer to the animal's natural surroundings than the relatively stark environment of the laboratory and animal house. Therefore the biological significance of such studies is unclear.

An approach which is more relevant to the rodent's natural lifestyle is the use of maze-learning. The rat is adapted to foraging and this biological predisposition can be exploited by the use of a maze such as the radial-arm (e.g. Olton, 1987). In this paradigm the apparatus consists of eight baited corridors radiating from a central chamber into which a rat is placed. The rat recovers the food rewards highly efficiently, visiting each arm of the maze only once. It appears that the animal navigates using spatial cues external to the maze and therefore builds up a "cognitive map" of the maze in relation to the environment. The processes involved in the paradigm seem to be different to that for avoidance learning (Staubli et al., 1984) with a central role for the hippocampus (Olton et al., 1986; O'Keefe and Nadel, 1978).

Research using the radial maze has led Olton to propose the existence of two distinct types of memory - working and reference memory. Working memory codes information about specific and temporal aspects of a situation and is dependent on the hippocampus. Reference memory, on the other hand, represents information about general rules and procedures specific to a task, is hippocampus-independent and may be processed in the neocortex.

The two forms of memory can also be distinguished in a Morris Water Maze in which a rat has to find a platform hidden in cloudy water (Morris et al., 1986, 1987). In this task the animal can use external visual clues (reference memory) or its own body movements (working memory).

Many theorists have postulated models in which two distinguishable types of memory operate in parallel. These include Olton's working and reference memory, Tulving's episodic and semantic memory, Squire and Cohen's declarative and procedural memory and Nadel's taxon and locale system. Recently attempts have been made to integrate these theories, for example Kesner (1986) has proposed the use of two broad terms defined as "data-based" and "expectancy-based" memory.

The use of mammal models in investigations of the neurobiology of learning and memory have contributed a great deal to our understanding of the types of memory which may be relevant to cognitive function in humans. However we are only beginning to understand the electrophysiological and cell biological substrates which underlie these processes.

1v) Long-term Potentiation.

One way in which studies of synaptic plasticity can take place in a well-controlled mammalian preparation is through the use of electrophysiological stimulation of brain loci which produce effects which share some similarity to memory formation. The most thoroughly investigated of such approaches is long-term potentiation, an approach which bears direct relation to some of the experiments undertaken in this thesis (see chapter 6).

Long-term potentiation (LTP) refers to an enduring increase in synaptic efficacy, measured as excitatory postsynaptic potential and/or population spike, of specific brain nuclei following tetanic stimulation of their inputs (Bliss and Lømo, 1973). LTP has been proposed as a possible mnemonic phenomenon for a number of reasons. The similarities between LTP and memory are summarised in the following section.

LTP has a long duration (days to weeks) following relatively short stimulation (seconds) (Bliss and Gardner-Medwin, 1973) and may therefore encode transient physiological events. The decay kinetics of hippocampal LTP, along with many of its biological concomitants, suggest that, like memory, it is multiphasic (Matthies, 1989). The induction of LTP may require more or less simultaneous (i.e. Hebbian) presynaptic and postsynaptic firing (Gustaffson and Wingstrom, 1988) and the physiological correlates of LTP appear to be restricted to tetanized synapses, there being no changes in the membrane potential, resistance or excitability of the soma.

Like some forms of learning, LTP can also be associative; if input is arranged to activate two adjacent, convergent pathways then there is greater resultant LTP in each single pathway than would be achieved by activation of only one pathway alone and weak stimulation, incapable itself of sustaining LTP will produce LTP if it is activated

concurrently with strong stimulation in a separate, converging pathway (Lee, 1983).

Like classical conditioning, associative LTP exhibits order dependency, LTP is only manifested if stimulation of the weak pathway occurs immediately before or concurrently with the strong pathway. These characteristics, and the importance of the hippocampus in many forms of learning (Thompson, 1986), have all lead to LTP becoming regarded as an important model in the study of the mechanisms of synaptic plasticity underlying learning and memory. Although LTP may not itself play any part in true learning and memory (discussed in detail below) it does have an important role in investigations of neural events which may be involved in synaptic plasticity.

One technical advantage of LTP is that it can be induced in hippocampal slices and thus the *in vivo* and *in vitro* correlates of the phenomenon can be elucidated. Perhaps its greatest contribution is in determining the types of events which may lead to the onset of plasticity.

LTP can be induced in many brain nuclei but is most commonly investigated in areas CA1, CA3 and the dentate gyrus of the hippocampus. In the following review of the biological concomitants of LTP findings from all three hippocampal subfields are treated together.

Although the biochemistry of LTP is as yet poorly understood, recent research has uncovered a series of presynaptic and postsynaptic events believed to be involved in the induction and maintenance of LTP. Briefly, tetanization results in elevated influx of Ca^{2+} ions into the neuronal spine, mediated by the voltage-dependent excitatory *N*-methyl-D-aspartate (NMDA) receptor channel. At resting membrane potentials, NMDA-linked ion channels are blocked by normal levels of extracellular Mg^{2+} ions (Mayer et al., 1984).

Other types of glutamate receptors - the monovalent cation channel-linked quisqualate and kainate receptors - are responsible for excitatory synaptic potentials (Collingridge et al., 1983).

The NMDA receptor is a complex with multiple binding sites and is associated with an ion channel. According to current models, the complex contains a recognition site for glutamate and allosteric binding sites for Mg^{2+} , phenylcyclidine (PCP) and glycine (Ascher and Nowak, 1987). Its associated ion channel is normally blocked by physiological levels of Mg^{2+} and, when open, allows influx of Ca^{2+} as well as flux of monovalent cations.

The glutamate recognition site is characterised by binding NMDA and is blocked by the specific antagonist, D,L-2-amino-5-phosphonovalerate (APV). Application of APV can block LTP but this inhibition is not universal - for example, APV does not block LTP produced by tetanization of the mossy fibre input to hippocampal subfield CA3 (Harris and Cotman, 1986).

When tetanization or simultaneous pairing of neighbouring synapses depolarise the postsynaptic membrane, the affinity of the NMDA-linked channel for Mg^{2+} falls and binding of glutamate to the receptor results in an influx of Ca^{2+} into the spine (MacDermott et al., 1986). The role of Ca^{2+} is crucial to the induction of LTP. Reduction of extracellular Ca^{2+} levels reversibly blocks the development of LTP (Dunwiddie and Lynch, 1979), as does intracellular injection of the Ca^{2+} chelator EGTA (Lynch et al., 1983). Transiently increasing intracellular Ca^{2+} results in an LTP-like phenomenon (Higashima and Yamamoto, 1985). LTP is also correlated with the amount of incorporation of radioactive Ca^{2+} (Bambridge and Miller, 1981) and with the number of electron-dense, Ca^{2+} -containing deposits in dendrites (Kuhnt et al., 1985).

Ca^{2+} could be responsible for a signalling mechanism in several ways. Firstly, calcium is involved in the activation of second messenger cascades. Ca^{2+} , in synergism with diacylglycerol, results in translocation of protein kinase C (PKC) from cytosol to the cell membrane (Bell, 1986). This translocation is believed to facilitate kinase activation or to allow access to substrates.

Following tetanization of the perforant path, there is a twofold increase in membrane-bound PKC and a corresponding halving in cytosolic levels (Akers et al., 1986). The involvement of PKC in LTP is further implicated by the finding that LTP-like phenomena can be induced by application of the PKC activators phorbol ester (Malenka et al, 1983) and oleic acid (Linden et al., 1986), although the potentiation achieved in these studies was shorter lived than is normally the case following tetanization.

The putative PKC substrate F1 (also called B50 or GAP43) undergoes changed phosphorylation during LTP (Routtenberg & Lovinger, 1985). The function of this phosphoprotein is unclear although it does appear to play a role in learning-related and development-related plasticity, possibly through its involvement in the mobilization of Ca^{2+} (De Graan et al., 1986) or in recycling of the presynaptic membrane (Scharma et al., 1986).

Type II Ca^{2+} /calmodulin dependent kinase (CaM kinase) may also play a part in LTP. When Ca^{2+} and calmodulin activate the CaM kinase, several brain proteins are phosphorylated and, importantly, the enzyme is autophosphorylated, resulting in Ca^{2+} -independent kinase activity (Thiel et al., 1988). Auto-phosphorylation and dephosphorylation by phosphatases may allow for a switch-like mechanism which could play an important role in LTP (Lisman & Goldring, 1988).

Another possibility is that Ca^{2+} may function in LTP by activation of Ca^{2+} -mediated proteases, including the neutral protease calpain I, resulting in hydrolysis of structural proteins including MAP2 (Siman et al., 1983) and spectrin (Siman et al., 1984). Hydrolysis of the submembraneous cytoskeleton of the dendritic spine could result in enlargement of the spine neck, thus allowing a spread of depolarisation or the insertion of more NMDA receptors (Kennedy, 1989). Calpain has also been reported to hydrolyse the regulatory subunits of both PKC and type II CaM kinase (Kwaitkowski & King, 1989) resulting in the generation of the active domains which would presumably function in the ways described above.

The picture for the maintenance of LTP is less clear although it is believed to involve changes in both pre- and postsynaptic mechanisms. Presynaptically, one of the most important tetanization-related events is an increased release of glutamate from nerve terminals (Bliss et al., 1986) and LTP results in an increase in presynaptic [^3H]glutamate concentrations following incubation of hippocampal slices with the labelled precursor [^3H]glutamine. Maintenance cannot, however, be solely attributed to increased transmitter release since analysis reveals that following LTP only the non-NMDA component of the EPSP is enhanced.

Because induction is believed to involve the predominantly postsynaptic events described previously and maintenance is probably a manifestation of both pre- and post-synaptic phenomena, there must exist some form of post- to pre-synaptic signalling mechanism. One possibility is that glutamate binds to presynaptic autoreceptors. It has been shown that in *in vitro* slices APV depresses the release of pre-loaded radioactive glutamate. Alternatively, there may be a diffusion of extracellularly secreted proteins from the post- to pre-synaptic membrane (Bliss et al., 1987; Duffy et al., 1981). Putative agents for this role include nitric oxide ("endothelium-derived relaxing factor") the production of which may be triggered by activation of the NMDA receptor (Garthwaite et al., 1988).

Arachidonic acid and/or its metabolites have also been proposed as possible retrograde messengers mediating the potentiation of transmitter release (Williams & Bliss, 1988).

At the postsynaptic membrane there is an enhanced sensitivity of the quisqualate and kainate (but not NMDA) type glutamate receptors (Muller et al., 1988). Postsynaptically, Ca^{2+} influx is responsible for the kinase cascades discussed above and may lead to multiple long-term changes within the cell.

The role of protein synthesis in maintaining LTP is implicated by the finding that pre-tetanzation injection of protein synthesis inhibitors (PSIs) such as anisomycin into the dentate area inhibit a late phase of LTP (i.e. ≥ 3 hrs) while leaving the earlier phase unaffected (Krug et al, 1984). In the CA1 of hippocampal slices the picture is somewhat different; PSIs applied pre- but not post-tetanzation inhibit LTP except for the first few minutes (Stanton & Sarvey, 1984).

Tetanzation also results in a transient increase in the formation of c-fos mRNA (Kaczmarek et al., 1989), indicating an activation of early gene expression. The role of protein synthesis in LTP will be discussed in more detail in later sections of this thesis. As yet, no signalling system which may activate genomic expression and link the induction and maintenance of LTP has been established.

There are several morphological changes which have been found following tetanzation. These include a number of changes in morphometric measures of the postsynaptic density (PSD) (Desmond and Levy, 1986a; 1986b). These results have direct relevance to results obtained in this thesis and are treated in detail in chapter 6.

An important question is, of course, how far is LTP actually related to learning. Barns (1979) found a correlation between the ability of rats to retain LTP and their memory of a spatial memory task. Older animals achieved lower scores than younger rats on both measures, suggesting the possibility of a common mechanism. In the same study, LTP abolished the normal spontaneous alternation in T-mazes in older rats while leaving younger animals unaffected, suggesting that LTP may saturate a finite pool of modifiable hippocampal synapses.

The NMDA antagonist APV, which disrupts LTP, also impairs spatial learning in a water maze (Morris, 1986). Comparable results were obtained when rats learned a spatial task in a multiple choice maze following tetanization of the perforant path. Recently acquired information and working memory were disrupted by LTP whereas established memory remained unaffected (McNaughton et al., 1986). In some learning tasks LTP may have opposite effects. For example, perforant path tetanization preceding discrimination conditioning of the nictitating membrane results in enhanced discrimination learning (Berger, 1984).

A more direct relationship between LTP and learning may be involved in certain situations. Tetanization of the perforant path after daily operant conditioning produced an enhanced population spike in trained animals compared to untrained controls (Skelton et al., 1987). In another experience-dependent study, rats transferred from an impoverished to a complex environment, which induces many memory-like processes, exhibit LTP-like changes in the dentate area. These changes subside after a time but reappear when animals are transferred to a new complex environment (Sharp et al., 1985).

Despite the above evidence linking LTP with learning, the exact relationship between the two remains unclear. LTP should probably be regarded as an analogue of learning in

which possible synaptic mechanisms of plastic changes and their maintenance may be elucidated.

CHAPTER 2.

2i) AVIAN LEARNING AND MEMORY.

Although birds have occasionally been elevated to honorary mammalian status (Thompson et al., 1983), the relevance of avian learning and memory to this thesis merits separate treatment. This section outlines some of the main findings of studies of song learning but will concentrate on a review of the current status of the neurobiology of learning and memory in the young chick. This will include data from studies using imprinting and, in particular, of a one-trial passive avoidance paradigm.

Song Learning.

Song learning is usually studied in canaries and finches. Song is produced by the syrinx, a structure located in the neck and innervated by the left and right hypoglossus nerves. These nerves are capable of functional regeneration; sectioning the left hypoglossus nerve resulted in profound vocalisation deficits whereas the same procedure on the right had far less effect (Nottebohm, 1972; 1984). If, however, the left hypoglossus was sectioned before Spring (i.e. before the song-generation season) the right hemisphere assumes dominance for song control. Further lesion studies identified the importance of the neostriatum and rostral hyperstriatum ventrale (HVc, later renamed the higher vocal centre) in song behaviour. Connections to this area traced by nerve degeneration indicated that the round nucleus of the archistriatum (RA) and area X of the parolfactory lobe are key loci in the control of song (Nottebohm et al., 1976).

Large sex differences in the morphology of the song-bird brain have been identified. Female canaries have a less well-developed area X compared to males, and in the female

zebra finch area X is non-existent while apparent in males. In canaries the size of the nucleus in both males and females was found to be correlated with singing behaviour (Nottebohm and Arnold, 1986). The sex differences in brain structures appear to be under the control of hormones; the growth of HVC and RA could be induced in females by the injection of testosterone (Nottebohm, 1980). The amount of testosterone in males undergoes seasonal changes which are correlated with changes in the size of testes, the relative volume of RA and HVC and with the size of song repertoire (Nottebohm et al., 1981).

The fluctuations in the size of song-related nuclei were interpreted as reflecting changes in the number of synapses in these regions. A greater number would facilitate connectivity between neurons, the formation of new neuronal networks and the representation of a more complex song repertoire (Nottebohm, 1984). Indeed, the volume of song nuclei was found to increase in the successive development from sub-song, through plastic song to full or stable song (Nottebohm et al., 1986).

Golgi studies of the hormone-sensitive type IV neurons of the RA in female canaries revealed that hormone-induced song development results in the addition of new synapses throughout the dendritic tree (Canady et al., 1988). This suggests that increased connectivity requirements may indeed underlie the increased volume of the nucleus.

The results of studies examining morphological differences in the brains of male and female songbirds pose interesting questions concerning the representation of information in the brain. Clearly the male does not have larger song nuclei to represent more information since the female must have equally complex representations in order to recognise and respond to the song. It appears that the morphological sex differences reflect the way in which stored information is utilised.

Although studies of the neurobiology of song-learning have revealed much about the neural substrates of the behaviour, the extent to which song generation and recognition can be regarded as learning is unclear.

2ii) LEARNING AND MEMORY IN THE YOUNG CHICK.

An alternative tactic to the study of behaviours such as song learning has been the use of imprinting and passive avoidance training in the precocial chick. The domestic chick, *Gallus gallus domesticus* is probably descended from the Burmese red jungle fowl and has retained many of the behavioural characteristics of its ancestor.

There are many advantages in using the chick for investigations of the neurobiology of learning and memory. They are cheap (no pun intended!), small and therefore easily housed in large numbers and are highly precocial being capable of independent exploration and learning within 12hrs of hatching. Young chicks have a minimal blood-brain barrier and a soft unossified skull facilitating the administration of pharmacological agents via the systemic or intracranial routes. Another major advantage of using young animals is that the "signal" of the memory trace is less likely to be lost in the "noise" of what the animal has already learnt about its environment. In this way the needle is hidden only in a haystack rather than in a needlestack!

2iii) Imprinting.

Imprinting is defined as when an animal, during sensitive periods, learns to restrict its preferences to a specific class of objects. The domestic chick is capable of both filial and

sexual imprinting, phenomena which occur at different sensitive periods (Vidal, 1980). A series of experiments from Horn and Bateson's laboratory in Cambridge has elucidated some of the biological consequences of filial imprinting in the domestic chick.

The imprinting paradigm employed is as follows. Chicks are hatched in the dark and are then exposed for a time to the imprinting stimulus. This may be a flashing coloured light, a rotating box or a stuffed jungle fowl. Imprinting is manifested by the approach behaviour of the animal. The amount of imprinting can be measured at test by placing the chick in a wheel-like cylindrical cage from which it can see the imprinting stimulus and an unfamiliar object. The cage is placed on a runway which includes a mechanism to move the bird away from the object which it attempts to approach and in this way the preference intensity is titrated. The more a chick attempts to approach an object, the further it is moved away from it. Eventually the "approached" object becomes so small in the animals field of vision that it attempts to approach the other object, when this becomes small enough in the visual field the chick reorients itself and once more attempts to approach the original stimulus. By this method a preference score can be calculated and the level of imprinting, assumed to be cumulative and saturable can be quantified by dividing the approach count to the imprinting stimulus by the total approach count.

One of the early results to emerge from these studies of imprinting was a correlation between the preference score to the imprinted stimulus and the degree of uracil incorporation into the forebrain roof of chicks. (Bateson et al., 1975). The incorporation of uracil was further localised by using "undertrained" and "overtrained" birds injected with radiolabelled uracil and then comparing the amount of uracil incorporation in serial brain sections. The animals were trained for different times on day one, on day two they were injected with the radiolabel and tested for an identical period. The undertrained animals had a greater amount of radioactivity in the intermediate portion of the medial

hyperstriatum ventrale (IMHV) than the overtrained birds. Compatible results were found by Kohsaka et al. (1979) who imprinted chicks on a floating red balloon, injected them with the metabolic marker, [14C]2-deoxyglucose (2-DG) and looked for localisation of radiolabel. The rationale for using this marker is discussed below in the section covering passive avoidance. Koshasha found that radiolabel accumulated in the lateral neostriatum of imprinted chicks and also in an area which overlaps with the IMHV. In a similar experiment, acoustically imprinted chicks were found to have increased amounts of 2-DG in an area which may overlap with the IMHV (Maier and Scheich, 1983).

The role of the IMHV in imprinting was further indicated by a series of lesion studies. Early studies by Salzen et al. (1975; 1978) indicated that crude lesions of the entire lateral forebrain prevented acquisition and retention of an imprinted preference for a yellow sponge or a stuffed green ball. The effect of lesions was later localised to the IMHV; bilateral lesions of this area prior to the completion of training prevented the development of a preference although birds were unaffected on visuomotor performance on a pecking task (McCabe et al., 1981). These results indicate that the IMHV is involved in acquisition of an imprinted preference. Lesions of the same area made after training and about 24 hours before the preference test had less effect but the lesioned chicks still showed reduced preference compared with sham-lesioned birds, unlesioned birds or birds with lesions of the hyperstriatum accessorium. This indicates that the IMHV is somehow involved in retention of an imprinted preference (McCabe et al., 1982). In the same study, cold-housed chicks were trained to peck at one of two patterns to receive a reinforcing jet of warm air. IMHV lesions had no effect on performance in this task.

In another investigation, chicks were trained to press one of two pedals which illuminated and rotated either a stuffed jungle fowl or a coloured box. Chicks learned to press the correct pedal and imprinted on the lighted stimulus. Chicks which had received bilateral

lesions of the IMHV performed normally on the pedal task but the preference for the familiar object was abolished (Johnson and Horn, 1986).

Such lesion experiments suggest that the IMHV is necessary for imprinting but not for attributing reinforcing qualities with stimuli or for associating operant responses with reinforcers.

Changes in a number of biological parameters are associated with imprinting. Morphological measures indicate that there is an increase in the length of postsynaptic densities (PSDs) in spine synapses of the left IMHV of imprinted birds (Horn et al., 1985). A corresponding increase in the number of NMDA receptors in the left IMHV has also been found (McCabe and Horn, 1988). These results are compatible with a model in which imprinting results in an enhanced efficacy of spine synapses of the left IMHV. The issue of functional asymmetry of the chick brain will be discussed later in this thesis.

Electrophysiological studies have revealed an inverse correlation between the mean firing rate of neurons and the approach activity of chicks imprinted on a red box but this has not been shown in animals exposed to a stuffed jungle fowl (Payne and Horn, 1984).

Certain pharmacological interventions have been found to effect imprinting. Injections of the noradrenalin antagonist DSP4 resulted in impaired imprinting to a box but not to a stuffed bird (Davies et al., 1985). Conversely, injections of testosterone enhanced imprinting on the fowl but not on the box (Bolhuis et al., 1986).

Although some interesting results have emerged from studies of imprinting, the relationship between imprinting and learning remains unclear. It is debatable whether a paradigm which depends on such a strong genetic predisposition can be regarded as real

learning. Additionally, as with all very young animals, the brains of chicks used in imprinting studies are in a state of dynamic flux and imprinting may be simply accelerating some component of the animal's development.

2iv) Passive Avoidance Learning.

The model of learning and memory which the Brain and Behaviour Research Group at the Open University routinely uses is passive avoidance learning in the precocial chick. The paradigm has remained largely unchanged in the twenty years since it was first used to study memory by Cherkin (1969) and is based on observations of the animal in the field (Morgan, 1896).

As part of its ontogenic development the young chick explores its environment by pecking at salient stimuli, particularly small, bright objects in its field of view, and thus learns very quickly to discriminate between food, pieces of faeces, pebbles etc. Passive avoidance training exploits this spontaneous behaviour by presenting experimental animals with a chrome bead coated with the bitter-tasting substance methylanthranilate (MeA). The bead is usually pecked within 10 seconds, the chick exhibits a typical "disgust response" (backing away from the bead, shaking its head and wiping its beak on the floor) and will avoid a similar but dry bead for at least 48 hours after the trial.

This behaviour exhibits the essential characteristics of memory: it is retained over time and is stimulus-specific in that the the animal will choose to peck at a green or a red bead if the other colour has previously been coated with MeA (e.g. Gibbs and Ng, 1979).

The passive avoidance paradigm has many advantages over other animal models. Firstly, events can be timed exactly since the training procedure involves a single event, that is,

pecking at a bead. Secondly, since the animal is very young at the time of training, the "signal to noise" problem is less than one might expect with older animals. Our research programme is also explicitly anti-reductionist, based on the belief that a true picture of the events which constitute memory formation and representation can only be gleaned through the integration of data from behavioural, physiological, morphological, biochemical and molecular biological concomitants of the training paradigm.

Rose (1981) defined the direction of the research programme by delineating six requirements of the necessary, sufficient and exclusive biological concomitants of memory formation. These are summarised as follows:

- 1) The mechanism should involve a neuroanatomically localised change in the level or rate of a biological measure during memory formation.
- 2) The time course of the biological change should mirror that of the memory phase in which it takes part.
- 3) "Non-specific" behaviours (stress, motor activity etc.) should not alone result in the biological change.
- 4) If the biological change is inhibited during the memory phase in which it is involved then memory formation should be inhibited, and vice versa.
- 5) Removal of the anatomical locus of change should result in interference of memory formation and/or recall, depending on the timing of ablation relative to training and testing.

6) Neurophysical recording from the locus of change should reveal altered cellular responses.

The Brain and Behaviour Research Group and other researchers have identified many consequences of passive avoidance training in the chick which are detailed in the following section. Unless otherwise stated, the changes in biological measures discussed below refer to significant effects found in birds trained on a methylantranilate coated bead (M birds) compared to controls which are presented with a water-coated bead (W birds). A detailed description of the training procedure is presented in section 4ii).

Because training on the passive avoidance paradigm involves a single, discrete event, it is ideally suited for the use of pharmacological agents to elucidate the exact timing of memory phases. Pretraining intracranial injections of ouabain (which inhibits the sodium/potassium pump) or the protein synthesis inhibitor, cycloheximide resulted in amnesia developing 10min or 30min post-training respectively (Gibbs and Barnett, 1976). The early, ouabain-insensitive memory phase lasting from the onset of training up until around 10min could be disrupted by injections of lithium chloride or potassium chloride (Gibbs and Ng, 1976).

These data led to the proposal of a three phase model of memory formation (Gibbs et al., 1978). Training leads to immediate post-tetanic hyperpolarisation, represented by changes in potassium conductance, underlying short-term memory for the first 10min following training. Intermediate term memory was postulated to be represented by hyperpolarisation due to changes in sodium pump activity and lasts from 10 to 30min post-training. The long-term, cycloheximide-sensitive memory phase develops after 30min and is believed to be based on the *de novo* synthesis of proteins.

The fact that disruption of short-term memory also disrupts intermediate- and long-term memory was interpreted as reflecting the necessity of earlier phases for the passage of a memory trace into later phases. This suggests that the memory phases are sequential processes with the memory trace being transcribed from one phase to the next.

Further evidence for this model comes from behavioural experiments in animals without pharmacological intervention. Populations of chicks trained in the passive avoidance task display two time-points of temporarily reduced retention. These "dips" in retention occur at 15min and 55min post-training and were interpreted as reflecting the transscription of the memory trace from STM to ITM and from ITM to LTM respectively (Gibbs and Ng, 1979). Interestingly the second retention dip shifted to 70min post-training if the chicks were housed and trained individually rather than in pairs suggesting that isolation stress alters the physiological constraints of LTM modulation (Gibbs and Ng, 1979).

The duration of the memory phases can be manipulated pharmacologically. The lengths of STM and ITM were extended and the time of LTM's susceptibility to protein synthesis inhibition was altered by the anti-epileptic drug, diphenylhydantoin (Gibbs and Ng, 1984a). Similar results were obtained using corticosterone and adrenocorticotrophic hormone₁₋₂₄ (Gibbs and Ng, 1984b) and with testosterone (Gibbs et al., 1986) suggesting that the duration of memory phases is under hormonal modulation.

The three phase model of memory formation has been verified and extended to some extent using intracranial injections of glutamate, ouabain and anisomycin (Patterson et al, 1986; 1988), with pre-training injections of these agents resulting in amnesia lasting from 0-5, from 10-20 and from 60-90min respectively. The main conflict between Patterson's and Gibb and Ng's results is that in her study the anisomycin-induced amnesia was not fully developed until 75min post-training, as opposed to 50min for Gibbs and Ng. This

apparent conflict was attributed to differences in the housing and training conditions in each study. In mammals, a similar three phase model of memory formation has been proposed to account for the timing of amnesia in rats following transient hypoxia (Frieder and Allweis, 1978).

Although the use of pharmacological interventions is clearly useful in delineating the phases of memory (see point 4 of Rose's scheme), the underlying biological processes which they inhibit are unclear without appropriate assays of nervous tissue. The following section reviews some of the experiments aimed at clarifying such mechanisms.

The glucose analogue 2-deoxyglucose (2-DG) is taken up by cells and begins to be metabolised as if it was glucose. However, it cannot pass completely through the glycolytic pathway and instead accumulates in metabolically active cells. A 2-DG-containing radioactive isotope was injected immediately before passive-avoidance training and the chick brain removed and sectioned 30minutes post-training, the radiolabel accumulated specifically in three forebrain loci. These were the medial intermediate portion of the hyperstriatum ventrale (IMHV), the paleostriatum augmentatum (PA) and the lobus parolfactorius (LPO) (Kossut and Rose, 1984). If the radiolabelled sugar was injected 5 minutes before, or 10 or 30 minutes after training, increased accumulation continued to be observed in the LPO when injected 10 minutes, but not 30 minutes, post-training. In the IMHV the accumulation persisted even when the injection takes place 30minutes post-training (Rose and Csillag, 1985). These results represent an increased metabolism that was taken to reflect neuronal activity in these three forebrain loci. The increased 2-DG accumulation in the IMHV was asymmetrical in that more radiolabel was found in the left IMHV than in the right. Many subsequent studies have indicated that brain function in the chick is indeed lateralised.

Which biological processes underlie the increased metabolism in these forebrain loci? One important change which was found to occur in the brains of trained chicks is an altered phosphorylation of a membrane-bound protein with an apparent molecular weight of 52kDa (Ali et al., 1988). The level of phosphorylation of this protein decreased significantly at 30minutes, but not at 10 minutes or 3 hours, post-training in isolated synaptic plasma membrane (SPM) fractions from the right, but not the left, forebrain roof. This asymmetric decrease occurred in SPMs but not in postsynaptic densities (PSDs), indicating a presynaptic locus of change since the SPM fraction contains the PSD as the major postsynaptic structure in the preparation. The 52kDa protein is a protein kinase C (PKC) substrate and has been found to be immunologically homologous to the B50/F1 protein discussed previously in relation to LTP (Routtenberg and Lovinger, 1983). The in vitro decrease in post hoc phosphorylation demonstrated by these studies is assumed to represent an increase of in vivo phosphorylation, the sites available for phosphate moieties being occupied as a result of the translocation of PKC from cytosol to membrane.

Immunological assays of membrane-bound and cytosolic PKC confirmed an increased membrane:cytosol ratio in the left but not right IMHV of trained birds (Burchuladze et al., 1990) and preliminary results indicate that this change may be primarily at the SPM.

In accordance with part 4 of the six point scheme proposed by Rose (1981), intracranial injections of PKC inhibitors caused amnesia. Inhibitors of PKC, such as mellitin, result in amnesia when injected within 5 minutes before or after training. The amnesia did not develop until 1.5-3hrs after training, that is, well after the significant change in phosphorylation, suggesting that the PKC-mediated phosphorylation of the 52kDa protein is involved in an enabling process necessary for the short-term memory to long-term memory transition.

Post-translational modifications of proteins such as phosphorylation appear to be involved in early phases of memory formation. The resulting modified proteins have half lives lasting from minutes to weeks whereas memory may persist for years. Several theories have been proposed to account for a more enduring cellular change which may maintain a memory trace immune to molecular turnover. Crick (1984) has proposed the existence of a dimeric phosphoprotein in which the phosphorylation state of each unit is independent. The kinases responsible for phosphorylation of each subunit were proposed to act only when the other subunit is in the dephosphorylated form. The post-translational modification would therefore persist despite molecular turnover.

A similar model has been offered in which a bistable molecular "switch" consisting of a kinase and a phosphatase exists (Lisman,1985). The catalysis of the kinase from the inactive to active state could be mediated by a different kinase or by the same kinase on a different molecule and inactivation would result from the action of the phosphatase. Modeling suggested that, given the correct balance of kinase and phosphatase, the "switch" could be maintained in an permanently active position.

Although these models present attractive hypotheses to account for an enduring memory trace, there is no experimental evidence for such persistent post-translational modifications being involved in memory formation or in neuronal function in general. It is more likely that the long-term changes in cellular properties allow cells to return to some "ground state" which maintains a memory trace through normal cellular "housekeeping" mechanisms.

The *de novo* synthesis of proteins is a general requirement of any mechanism involving cellular reconstruction as a concomitant of the memory trace. The role of protein synthesis in the establishment of long-term memory has been thoroughly investigated both in the chick and in other preparations (Reviewed in Davies and Squire, 1984). In addition to

findings from the use of intracranial injections of protein synthesis inhibitors discussed above with reference to memory phases, the incorporation of radioactively labelled leucine into soluble proteins of the forebrain roof was found to increase from 30min up to at least 24 hrs following training. In particular there was an increase of leucine incorporation into the major cytoskeletal protein tubulin as well as an increase in absolute levels of detectable tubulin (Mileusnic, et al., 1980). The results of this and related studies will be discussed in detail in chapter 5.

Complementing these studies, recent experiments have revealed that there is an increase in the expression of the proto-oncogene, c-fos, following passive avoidance training (Anokhin et al., 1991). Oncogenes encode nuclear proteins which affect transcription or mRNA processing. The proteins are short-lived but appear to be involved in the induction of enduring cellular changes (e.g. Weinberg, 1985; Halazonetis et al., 1988). These genes have been reported to be induced by ion fluxes, transmitters and second messenger systems of the kind described during the early phase of memory formation. This makes them ideal candidates for involvement in type of changes which make memory traces permanent and immune to molecular turnover. It should be noted that the exact nature of the altered cellular state is unknown, as is the nature of the message which converts short-term modifications in neuronal circuitry into long-term memory traces.

The involvement of glycoproteins in memory formation has been of particular interest to the Brain and Behaviour Research Group. This is because they are abundant in the plasma membrane and potential changes in their carbohydrate moieties could allow for subtle alterations in cellular signalling which may play a part in memory representation.

One of the major carbohydrate sugar units is fucose. Radiolabelled fucose, injected intracerebrally, showed an increased incorporation into SPM glycoproteins of the

forebrain roof of trained birds for at least 24hrs following training (Sukamar et al., 1980). Levels of the relevant kinase, fucokinase, were also elevated (McCabe and Rose, 1985) and the increased fucose incorporation was unaffected by the protein synthesis inhibitor cycloheximide, indicating that training results in a post-translational increase of fucose incorporation. These experiments further revealed that, in addition to the increased incorporation of fucose into membranes of the forebrain roof (the area containing the IMHV), there was an elevated uptake into the right forebrain base (which contains the PA and LPO).

An elegant experiment using brief, sub-convulsive transcranial electroshock revealed that the increased fucose incorporation was not due to non-memory related consequences of the training procedure such as stress or increased motor activity. When shock was administered immediately following training, birds were amnesic and no elevation in [^3H]-fucose was detected. If the shock was delayed, however, no amnesia was evident and the increased fucose incorporation into the forebrain base and anterior forebrain roof persisted (Rose and Harding, 1984).

The incorporation of fucose into glycoproteins is inhibited by the sugar 2-deoxygalactose (2-D-gal) which competes with preterminal galactose, thus preventing the addition of the final fucose. Intracranial injections of 2-D-gal, when administered within a time window 2hrs before to 2hrs after training, resulted in amnesia for the passive avoidance task. More recently, Rose and Zamani (1990) have demonstrated that there is a second time window of susceptibility to 2-D-gal which reaches a maximum around 6hrs post-training. These data, along with the forebrain base results, are relevant in the light of recent studies using electrolytic lesions of discrete forebrain loci.

Bilateral, pretraining lesions of the IMHV resulted in amnesia for the passive avoidance task (Davies et al., 1988) indicating that some aspect of memory formation (acquisition, retention or recall) requires the intact IMHV. Importantly, however, bilateral post-training lesions of the same area (made 1hr or 6hrs following training) did not result in amnesia (Patterson et al., 1990), demonstrating that the IMHV plays a necessary role in the acquisition phase of memory but not in storage or recall. The site of acquisition could be further localised to the left IMHV since unilateral pre-training lesions of this area resulted in amnesia whereas lesions of the corresponding right IMHV were not amnesic (Patterson et al., 1990).

The IMHV is clearly not involved in retention, at least 1hr and thereafter following training. As mentioned above, there is data implicating the role of the forebrain base in memory formation. Studies of song learning in other avian species have identified the involvement of "area X", a forebrain base locus which shares some homology with the chick LPO, one of the areas which "lights up" in 2-deoxyglucose mapping following chick passive avoidance. Based on these observations, Gilbert et al. (1990) investigated the effect of LPO lesions, either alone or in combination with IMHV lesions, using our passive avoidance paradigm. Unlike the picture for the IMHV, bilateral pre-training lesions of the LPO were without effect on passive avoidance. However, bilateral LPO lesions administered 1hr post-training did render birds amnesic when tested 24hrs after training. It also seems that for memory to persist any part of the LPO is sufficient since unilateral ablation of either hemisphere's LPO was without effect on memory for the task.

The lesion studies suggest a model in which the left IMHV is necessary for the acquisition phase of memory for the one-trial passive avoidance task whereafter the LPO, perhaps in conjunction with other loci, is necessary for the retention of that memory. These data have

been interpreted as assigning a role to the LPO in a secondary memory store called S' by Horn (Cipolla-Neto et al., 1982). However S' was shown to require an intact right IMHV during imprinting whereas in the passive avoidance paradigm an intact right IMHV does not appear to be necessary. This may simply mean that different mechanisms are used for imprinting and passive avoidance.

The results should, in any case, be regarded with a certain amount of caution; lesion studies may not be providing information regarding the role of brain nuclei but about how the remaining, unlesioned portion of the brain operates in the absence of the missing nucleus.

Electrophysiological studies have revealed that there is a dramatic increase in spontaneous "bursting" activity recorded in the IMHV up to 12hrs following passive avoidance training (Mason and Rose, 1987). Increased bursting was demonstrated to be a true concomitant of memory formation using the electroshock treatment described in relation to the fucose incorporation experiments. Brief, subconvulsive transcranial electroshock immediately following training was amnesic and abolished the increase in bursting, when the application of shock was delayed the memory for the task persisted and so did the increase in bursting (Mason and Rose, 1988).

Much morphological data exists regarding changes in the chick brain following passive avoidance training. Measures taken at 24hrs post-training at the level of the electron microscope have revealed morphological differences between hemispheres and between M and W birds. Measures in the IMHV of M chicks indicated a 22% increase in the presynaptic bouton density in the left hemisphere compared to the right which was not evident in W birds. In W birds there were 12% more vesicles per unit volume of neuropil in the right compared the left IMHV, the opposite was the case for M birds. The mean

length of postsynaptic thickening was 12% greater in the right IMHV compared to the left in W chicks and this asymmetry was abolished in M-trained animals. The most dramatic result reported was that, in M chicks, the left IMHV contained 61% more vesicles per synapse than the right - a difference which was not apparent in W controls (Stewart et al., 1984).

Similar studies were undertaken in the LPO. There was a 59% increase in the numerical density of synapses of both left and right LPOs of W compared to M chicks. The synaptic thickening was 10% greater in the right compared to the left LPO of W chicks while the reverse was found for M birds. No differences were found in the volume density of presynaptic boutons or in the mean bouton density when between-hemisphere or between-training group comparisons were made. Both the numerical density of synaptic vesicles and the number of vesicles per bouton was 50% greater in the left LPO of M compared to W chicks. No differences were found in measures of mean synaptic curvature, mean length of postsynaptic thickening, numerical density of synaptic synapses or the volume density of presynaptic boutons. The mean bouton volume was greater in the left LPO of M chicks compared to W-trained controls. The numerical density of synaptic vesicles, and the number of vesicles, per presynaptic bouton was 15% greater in the right LPO compared to the left in W birds, training on the M bead appeared to abolish this asymmetry (Stewart et al., 1987).

As well as the studies using electron microscopy, the IMHV has been studied at the level of the light microscope. Approximately 24hrs following training, M birds were found to have a higher dendritic spine density in both the left and right IMHV. This change exhibited hemispheric asymmetry with a larger increase in the left IMHV. This change was accompanied by an increase in measures of spine head diameter and a decrease in spine stem length of M birds (Patel and Stewart, 1988). The relation of the change in

spine density to memory was investigated by rendering a group of birds amnesic through the use of a brief transcranial subconvulsive shock. Non-amnesic M birds again displayed the increase whereas amnesic birds' spine density was similar to that observed in water controls (Patel et al., 1988). This implies that the increase in spine density has a direct role in memory formation, possibly by providing a greater number of synaptic contacts in the formation of neuronal circuits.

The results of the above studies have been synthesised and presented as a model of memory formation (e.g. Rose, 1991). The model suggests that in the first seconds and minutes following training, there is pre and postsynaptic depolarisation within the cell assembly which represents a memory trace. The depolarisation may be a phenomenon similar to the post-tetanic effects observed with respect to long-term potentiation and is postulated to be mediated by changes in the flux of ions such as Ca^{2+} across the synaptic membrane. At these early time points 2-deoxyglucose mapping indicates that there is increased metabolic activity in the IMHV, PA and LPO.

These very early events lead to a number of changes within cells which occur from minutes to hours following training. In the IMHV, PKC is translocated to the cytosol to the presynaptic membrane where it catalyses the phosphorylation of B50. The precise role of this phosphorylation is unknown but appears to be involved in an enabling process within the transition from short to long-term memory.

At similar time-points, there is an increase in the expression of c-fos in the IMHV and LPO and a consequent synthesis of new proteins. The signal which converts synaptic changes into genomic activation is unknown but may involve changes in calcium flux and phosphorylation similar to those observed at the synapse.

During this phase there is a transient increase in receptor binding which may reflect changes in the efficacy of synapses within the circuit. A change which may be related is an increase in neuronal bursting which has been interpreted as indicating an increase in the amount of "active" membrane (Mason and Rose, 1987).

By 24hrs following training, a number of morphological changes have occurred in the chick brain. Morphometric changes which may reflect alterations in the efficacy of neuronal circuits include increases in the number of synaptic vesicles in the left IMHV and left LPO and, in the same areas, postsynaptic increases in synaptic apposition length zone. In the IMHV there is an increase in dendritic spine density and in spine head diameter.

Events which appear to be involved in long-term synaptic remodelling during memory formation include the synthesis of new proteins. These include tubulin, a cytoskeletal protein which would be expected to be involved in the axonal and dendritic transport of the constituents of synaptic remodelling as well as being incorporated in modifications of the synaptic cytoskeleton itself (see chapter 5).

At the synapse there is an increase in the incorporation of subunits into glycoproteins which are postulated to be involved in connectivity changes within a neuronal circuit. There appears to be two waves of glycoprotein synthesis; one occurring at the time of training and the other taking place some hours later. The second wave of glycoprotein synthesis has not been localised but may take place in the forebrain base, the area containing the LPO and which appears, from the evidence of lesion studies, to be involved in the maintenance of the memory trace.

Although the model presented above describes a fairly coherent picture of the events which constitute changes in synaptic connectivity and signalling, it is by no means complete. There are large gaps in our knowledge of how the components of memory phases are transcribed from one phase to the next. The way in which neurobiological processes with very different time-courses are expressed as distinct memory phases is unclear. Perhaps more importantly is the lack of theory, let alone data, pertaining to the rules by which changes observed at the cellular level are translated into the behavioural expression of memory (Rose, 1988).

2v) THE USE OF ANTIBODIES TO STUDY SYNAPTIC PLASTICITY.

The acceleration of development and refinement of immunological techniques over the past decade or so means that such methods may now be applied to the study of learning and memory. The sensitivity and selectivity of antibodies means that they particularly lend themselves to the study of plasticity in two ways. Antibodies can be administered by intracranial injection to try and disrupt specific phases of memory formation and they can be used to map changes in specific antigenic proteins following events which result in plasticity.

One of the first reported examples of the effect of antibodies on neural function comes from Pasteur (1885). He found that patients who were vaccinated with rabies virus grown on rabbit spinal cord exhibited neural disfunction believed to result from the production of brain iso-antibodies. Other early studies used antibodies to demonstrate the organ specificity of brain antigens (Witebsky and Steinfield, 1928). This was soon followed by the use of anti-caudate and anti-hippocampal serum to distinguish between white and grey matter (Reichner and Witebsky, 1934).

The possibility of using antibodies to disrupt neural function was given impetus by the finding that the auto-immune disease, allergic encephalomyelitis, could be produced experimentally in animals (Morgan, 1946; Kabat et al., 1946). By the early 1960s antibodies were being used to try and disrupt neural function, for example electroencephalograph activity and behavioural measures of the cat were found to be profoundly altered by intraventricular injection of anti-brain antibodies (Mihailovic and Jankovic, 1961).

Although there was an abundance of studies using the techniques of immunology to study changes in behaviour in the 1970s, like many investigations of neural function at this time they are memorable for their lack of biochemical and/or behavioural rigour.

Recently there has been a re-emergence of such research which has highlighted the potential for the use of immunological techniques in neurochemistry and, in particular, studies of changes in behaviour. Jankovic (1985) lists seven problems which can now be effectively addressed within the emerging discipline which he terms "immunoneurology". Of special relevance to this study he includes the "structural and functional dissection of the neuron, brain and mind by means of immunological agents, and antibrain antibodies in particular".

Many studies have used antibodies to characterise synaptic structures and a few examples, selected from a large body of literature are presented here for illustration.

The cellular localisation of the brain specific proteins S-100 and 14-3-2 have been investigated using antisera (Cicero et al., 1970). The soluble, acidic proteins S-100 and 14-3-2 (M.W. 24kDa and 40kDa respectively). were studied during lesion-induced cell degeneration of the cerebral cortex; the immunoreactivity of 14-3-2 declined progressively whereas that of S-100 increased. The authors interpreted this as suggesting a primarily neuronal location for 14-3-2 with S-100 being associated with glia. The neuronal specificity of 14-3-2 has been confirmed immunologically and has been reported to be a major constituent of synaptosomes (Grasso and Chen, 1974).

Stuhlfauth and Seeds (1983) raised antisera to map the development of SDS-PAGE separated cerebellar membrane proteins which appeared to be cell-specific or developmentally regulated. Immunological staining of frozen sections and dissociated cell

cultures of cerebellum showed some antibodies binding specifically to Purkinje cells or Purkinje cell bodies while others exhibited a pattern characteristic of a synaptic localisation. Cerebellar membranes from mouse behavioural mutants *reeler*, *staggerer*, *nervous* and *weaver*, were found to exhibit different patterns of antibody staining on Western Blots when compared to normal mice.

The relative resolving power of monoclonal antibodies is demonstrated by Kushner (1984) who raised a library of 141 monoclonal antibodies to *Torpedo* cholinergic synaptosomes. Of those tested, most were synaptosome-specific, 61% did not cross-react with brain homogenate and only about 11% cross-reacted with liver. Many of the monoclonals cross-reacted with nervous tissue from frog and rat, reflecting the high degree of neural conservation between species.

Antibodies have been used to disrupt neural function in a variety of situations. A polyclonal antibody raised against a 50kDa protein secreted by denervated rat muscle was found to suppress sprouting at the neuromuscular junction (Gurney, 1984). The antibody depressed sprouting at the denervated junction by approximately 50% compared to controls.

Effects of antisera have also been reported on brain electrogenesis as measured by electroencephalograph (EEG) activity. Intraventricular infusion of anti-hippocampal antiserum resulted in a large increase in the theta rhythm of EEG over the seven hours following antibody infusion. A similar, though less dramatic effect was observed with anti-S-100 (Shtark et al., 1987). The same authors also examined the effects of antisera on resting potential and action potential in snail neurons. The effect of application of both antisera was to depolarise the membrane with the resting potential decreasing for 10 to 15mins before returning to a constant normal level. The size of the action potential was

reduced to 60% that of the control level in the presence of antibody. In the same study a rather strange "learning" paradigm was also examined. Rats' hind legs were shocked if an electrophysiological parameter - the "average level of the right hippocampal EEG asymmetry" - was exceeded. Anti-brain antiserum inhibited the ability of animals to learn as determined by the number of passages through this threshold.

Previous immunological work in our laboratory has resulted in the production of a monoclonal antibody (411B) which recognises a 170kDa epitope highly enriched in PSDs (Bullock et al., 1988). 411B has been used to map developmental changes in the epitope (Bullock et al., 1987), this latter study found a small peak in antibody binding to whole forebrain homogenates at 4 days post-hatch followed by a higher peak between 8 and 14 days post-hatch. Interestingly, the antibody did not show training-induced changes in binding to brain homogenates taken 24hrs after training (Bullock et al., 1987), whereas monoclonal antibodies to synaptic vesicle-specific proteins (anti-p65 and anti-SV2) used in the same experiment exhibited hemispherically asymmetrical reductions in binding to two of the three forebrain nuclei examined.

411B cross-reacts with rat PSDs and increased in binding to homogenates of the corpus striatum and hippocampus when the animals exhibited haloperidol-induced dopamine supersensitivity (Loessner et al., 1988), lending support to the hypothesis that this phenomenon results from changes in postsynaptic plasticity.

The value of 411B as a marker for postsynaptic plasticity was given further impetus by the finding that long-term potentiation results in an increase in the binding of 411B to homogenates of the target, dentate gyrus 8hrs following tetanisation. The findings from experiments with 411B will be discussed in more detail in chapter 6.

A number of studies have used antibodies to disrupt memory formation, maintenance or recall in various paradigms. Immunological methods have also been used, to a much lesser extent to probe for neural changes following memory formation. These studies are reviewed in the introduction to chapter 4.

Against a background of a developing body of work utilising antibodies to delineate processes involved in memory formation, the aim of this project was to develop and use immunological techniques to map changes in neural antigens as a consequence of synaptic plasticity. Within this rather broad remit, the specific aims were as follows.

The first aim was to raise antibodies to synaptic structures believed to be involved in memory formation, the postsynaptic density was chosen as the immunogen for this purpose. Anti-PSD antibodies were used to attempt to disrupt memory formation in our chick passive avoidance paradigm. Following from this, we wanted to determine if immunological methods could be used to identify both the temporal and brain-regional parameters of amnesia.

Secondly we hoped to use the methods of immunology to try to identify changes in neural antigens in the hope that these techniques may reveal time- and loci-specific changes in such molecules which may not be apparent using less sensitive methods. For these studies monoclonal antibodies to the cytoskeletal proteins α -tubulin and microtubule associate protein 2 (MAP 2) were used.

Thirdly, we wished to develop further collaborative studies between our own department and the laboratory of Professor Bernd Loessner in Magdeburg, Germany which has revealed, through the use of antibodies, changes in immunoreactivity following LTP. It was hoped that, using antibodies directed towards antigens which are synaptically

enriched in the rat, it may be possible to define the detailed temporal and loci-specific changes following tetanisation

The principle aim of this project was to use antibody technology to study synaptic and neural plasticity. This necessitated examining various types of biological change in different paradigms which are used in the study of plasticity. The background to each set of experiments will therefore be discussed in detail in the introduction and discussion sections of the relevant chapters (4, 5 and 6).

CHAPTER 3: PRODUCTION AND CHARACTERISATION OF POLYCLONAL ANTIBODIES TO CHICK FOREBRAIN POST-SYNAPTIC DENSITIES

INTRODUCTION.

Part of the rationale behind this thesis was to attempt to assess the potential amnesic effect of antibodies injected intracranially into the chick brain. The postsynaptic density (PSD) fraction was chosen as the immunogen to produce such antibodies, the reason for this choice being threefold. Firstly, the PSD contains proteins likely to play a functional role in synaptic plasticity and so the binding of antibodies to any of these proteins has the potential for producing an amnesic effect. Secondly, the chick PSD has an antigen composition which is identifiable, or at least not as complex as other less "specialised" fractions which precede the PSD in the preparation procedure. The third reason for this choice of immunogen is technical; the isolation of the PSD fraction is relatively straightforward and well established in our laboratory and the quality of the fractionation does not vary significantly between preparations.

The following sections describe preparation of PSDs, the immunisation protocol and basic characterisation of resulting antisera. The characterisation includes the enzyme-linked immunosorbent assay (ELISA) and the Western Blot which are major methodologies referred to throughout this thesis, they are therefore detailed below and deviations and changes from this general method will be indicated where relevant.

METHODS.

3i) Preparation of SPM and PSD Protein.

Postsynaptic densities (PSDs) were isolated according to the method of Murakami et al (1986), all steps being carried out at 4°C unless otherwise stated. Day-old chick forebrains were quickly removed and their wet weight recorded. Forebrains were homogenised (10% w/v) in 0.32M sucrose/ in 2mM HEPES, pH 7.4 in a teflon-glass homogeniser (700rpm; 8 up and down strokes). Homogenates were centrifuged at 1,000gmax in a Beckman J2-21 centrifuge for 5 minutes, to give supernatant, S₁, containing cell fragments, mitochondria and synaptosomes. The pellet was washed in 0.32M sucrose in 2mM HEPES, pH 7.4 and recentrifuged as above. The second supernatant was combined with S₁ and centrifuged at 15,000gmax to give pellet P₂, the crude mitochondrial fraction. The supernatant (microsomes) was discarded and the pellet was washed X2 (20min; 15,000gmax) in 0.32M sucrose/ 2mM HEPES, pH 7.4 to further reduce microsomal contamination (Gurd et al., 1974). The pellet was resuspended in lysis buffer (5mM Tris/HCl, 50µM Ca²⁺, pH 8.1), at a concentration of 10ml buffer per gram wet weight of original tissue and incubated on ice for 30 minutes. The resulting lysate was pelleted to give P₃ (membranes) by centrifugation at 120,000gmax for 20min in a Beckman L8-55 ultracentrifuge and resuspended in lysis buffer (3ml per gram wet weight tissue) using a glass Dounce homogeniser. 0.4ml per wet weight tissue of INT solution was added (2.53mg/ml p-iodonitrotetrazolium violet, 65mg/ml sodium succinate, 0.2M sodium phosphate, pH 7.4) and diluted to 4.5ml per gram wet weight tissue with 0.2M sodium phosphate, pH 7.4 and incubated for 30 minutes at 25°C. During this incubation the INT acts as an electron acceptor in the mitochondrial oxidation of succinate by succinate dehydrogenase. Under these conditions this results in the accumulation of a purple formazan precipitate within mitochondria, increasing their density relative to

SPMs and facilitating mitochondria-SPM separation (Nieto-Sampedro et al., 1981). The solution was centrifuged at 77,000gmax for 15 minutes, washed in 0.16M sucrose in 2mM HEPES, pH 7.4, and repelleted.

P₃ was fractionated on a flotation/sedimentation gradient. A discontinuous sucrose density gradient was set up as follows: pellet P₃ was resuspended in 0.25ml/gram wet weight tissue of lysis buffer in a glass Dounce homogeniser. 1.3ml/ gram wet weight tissue of 48% (w/v) sucrose/2mM HEPES, pH 7.4 was added and the suspension dispersed, 28.5% (w/v) sucrose, 2mM HEPES, pH 7.4 was layered onto the suspension to a final total volume of 17ml. The gradient was centrifuged at 130,000gmax for 75min. During centrifugation myelin, because of its relatively low bouyant density, migrates to the top of the gradient, INT-containing mitochondria form a tight pellet and SPMs accumulate at the interface between the two sucrose solutions. The SPMs were carefully removed, resuspended in 50µM Ca²⁺ in distilled water and pelleted at 150,000gmax for 20 minutes and resuspended in a small volume (0.5 - 1.0ml) of 50µM Ca²⁺.

The protein concentration of the SPM fraction was determined using a modification of the Bradford method (Bradford, 1976) using commercial Bovine Serum Albumin as a standard. The PSD fraction was isolated from the SPM fraction using the mild, non-ionic detergent octyl glucoside (OG) and aqueous two-phase polymer partitioning (Gurd et al., 1982; 1983). Between 3mg and 8mg of SPM protein was loaded onto a polyethelyne glycol/dextran gradient containing the following: 0.5ml of SPM protein; 0.5ml of 0.5M NaHCO₃, pH 9.6; 100µl of 25% (w/v) octyl glucoside; 100µl of distilled water; 1.25g of 20% w/v polyethelyne glycol 600; 1.5g of 25% (w/v) dextran, made up to a total final weight of 5g with 50µM Ca²⁺ in distilled water. The mixture was dispersed by vortex mixing, centrifuged at 1,000gmax for 7.5min in an MSE Coolspin centrifuge and the PSD layer removed from the interface between the polyethelene glycol and the dextran.

The PSDs were resuspended in Tris-buffered saline/50 μ M Ca²⁺, pH 7.4, and pelleted at 14,000gmax for 10min. The protein yield of the PSD fraction was determined and PSDs were stored frozen in 100 μ g aliquots prior to use.

3ii) Morphological Characterisation of PSDs.

The relative purity of isolated SPMs and PSDs was characterised morphologically using electron microscopy. The fractions were pelleted by centrifugation at 14,000gmax for 15 - 20min. Supernatant was removed and the pellet was fixed by saturation and rotation for 10min with 2.5% gluteraldehyde and 0.5% paraformaldehyde in 0.1M cacodylate buffer, pH 7.5. The pellet was then fixed in 1% osmium in the same buffer.

The fixed block was washed and stained with saturated uranyl acetate, dehydrated by incubation in a successively increasing percentage ethanol series and embedded in araldite. The block was then stained with lead acetate and sections of 50 - 70nm thickness were cut. The sections were viewed in a Phillips 100 electron microscope at 60 - 80kV.

Results.

Representative fields of two PSD fractions from which immunogen was prepared are presented in Figure 3i) as they were observed under the electron microscope. The fraction is highly enriched in PSDs (indicated by arrowheads) although there is evidence of contamination possibly arising from cell membrane debris.

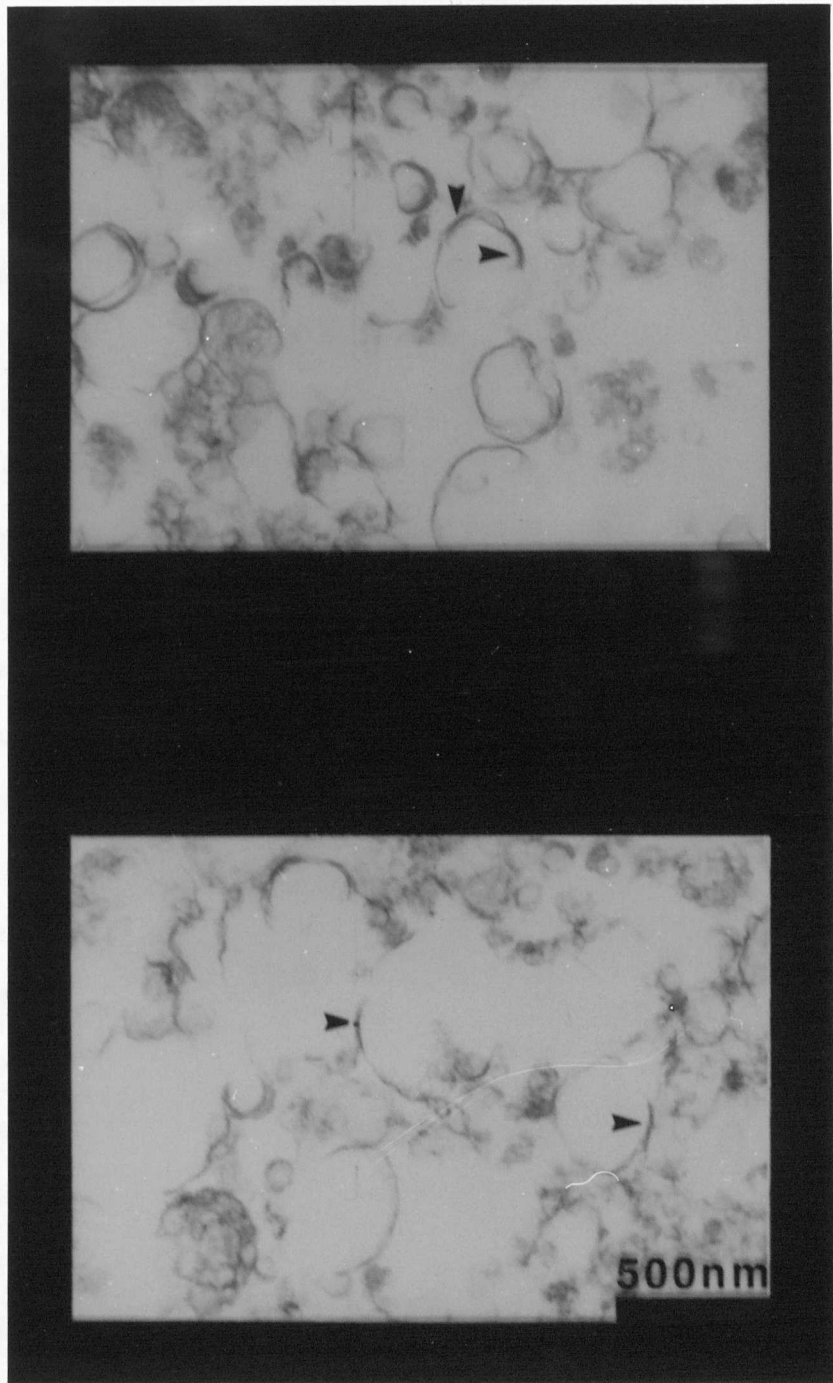


Figure 3i) Representative fields of postsynaptic densities (PSDs) as viewed under the electron microscope. PSDs are indicated by arrowheads.

3iii) Immunisation Protocol.

Prior to immunisation of animals, several bleeds were taken to provide pre-immune serum. This is because animals may receive other antigenic challenges in addition to those of the immunisation regime and in later experiments it was essential to ensure that only the immunogen-related antibodies were responsible for experimental results.

Rabbits were injected with PSD protein following a standard immunisation protocol. Injections were of 50 - 100µg of PSD protein suspended in 0.5 ml of phosphate buffered saline and emulsified with an equal volume of Freund's adjuvant. Immunogen preparations were administered by multiple subcutaneous injections. The first four injections were administered weekly in Freund's Complete Adjuvant, followed by similarly spaced injections in incomplete adjuvant. After 6 such immunisations the rabbits were alternately bled then boosted with 50µg - 100µg of protein approximately weekly.

The blood from each bleed was allowed to clot and the serum removed. Using this protocol 6 polyclonal antibodies were raised; 4 to PSDs and 2 to SPMs. To ensure that antibodies had been successfully raised using PSDs as an antigen, Laurell rocket immunoelectrophoresis was performed on the antiserum. Enzyme-linked immunosorbent assays (ELISAs) were performed to ensure that the titre of the antiserum remained high throughout the immunisation period and the antibody was titrated using the same enzyme-linked immunosorbent assay (ELISA) protocol. The molecular weights of the epitopes to which the antibodies bind was determined using SDS PAGE followed by Western Blotting. One of the anti-PSD sera (from rabbit number 14 and named R14) thus obtained has been characterised in a number of ways.

3iv) Rocket Immunoelectrophoresis.

Laurell Rocket immunoelectrophoresis was carried out on serum from the first bleed following immunisation to ensure that rabbits injected with PSD protein did produce antibodies. A 2% (w/v) aqueous solution of agarose was liquified by heating over a Bunsen Burner for one hour then added to an equal volume of gel buffer (1.107g/l diethylbarbituric acid; 7.007g/l sodium barbitone; 1.024g/l calcium lactate, pH 8.6). The mixture was allowed to cool to approximately 50°C and 5% (v/v) serum was stirred in. The serum-agarose mixture was poured onto 76mm X 26mm microscope slides (12ml/plate) and allowed to set, 50µg of PSD protein in 50µl of tris-buffered saline (TBS) was added to a 5mm diameter well and electrophoresed at 10V DC (about 40mA) overnight. The slides were stained with Coomassie blue and destained to reveal immunoprecipitation lines.

Results.

Typical rocket immunoprecipitation results are shown in Figure 3ii). Clear lines of precipitation are visible, indicating the presence of antibodies to certain PSD proteins.

3v) Enzyme-linked Immunosorbent Assay (ELISA).

The procedure for Enzyme-linked Immunoabsorbant Assay (ELISA) was according to the following procedure (from Engvall and Perlmann, 1971). All steps were carried out in a humidified chamber and, unless otherwise stated, at room temperature. Plastic, 96-well, flat-bottomed microtest plates were primed by a 1hr incubation with 100µl/well of

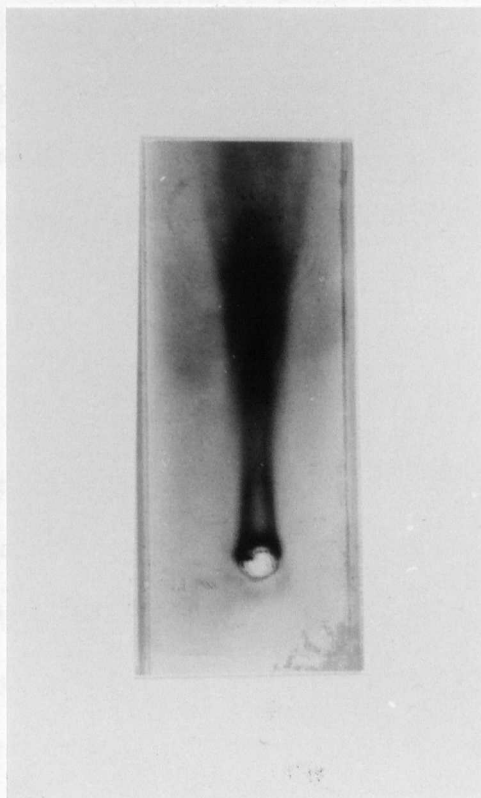


Figure 3ii) Representative rocket immunoelectrophoresis of R14 using PSD as antigen. Clear lines of immunoprecipitation are visible.

coating buffer (0.1M carbonate/bicarbonate, pH 9.6). The buffer was decanted and the plates were blotted against a tissue paper pad until no evidence of buffer remained in any well. Antigen (50µl/well) was added at appropriate concentrations and allowed to bind to the plastic overnight. The wells were washed X5 with washing buffer (Phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20) and residual binding sites were blocked by a 1hr incubation with 100µl/well of blocking buffer (PBS, pH 7.4, containing 0.05% Tween 20 and 3% Bovine Serum Albumin). 50 µl/well of antibody at an appropriate dilution in washing buffer was added and allowed to bind overnight. The plates were washed X5 and second antibody at a dilution of 1:500 in washing buffer was added. The second antibody was affinity purified F(Ab)₂ fragments of goat anti-rabbit IgG or goat anti- mouse IgG (gamma and light chain specific), as appropriate, linked to horseradish peroxidase.

The plates were incubated for 2hrs at 37°C and washed X5. The substrate used was 1mg/ml *o*-phenylene diamine added at 50 µl/well in 0.1M citrate buffer, pH 5.0. This was incubated in the dark at 37°C for approximately 20 minutes while the colour developed and then the enzyme-substrate reaction was stopped by the addition of 50µl/well of 2M H₂SO₄. Absorbances of each well were read at 492nm using a Titertek Multiskan plate reader.

3vi) Levels of Anti-PSD Antibodies over Time.

The above procedure was used to ensure that serum from successive bleeds over an 8 month period after the onset of immunisation continued to contain antibodies to PSDs. 50µl of PSD protein was added to each well at a concentration of 50µg/ml and R14 serum from inter-immunisation bleeds was used at a dilution of 1:500.

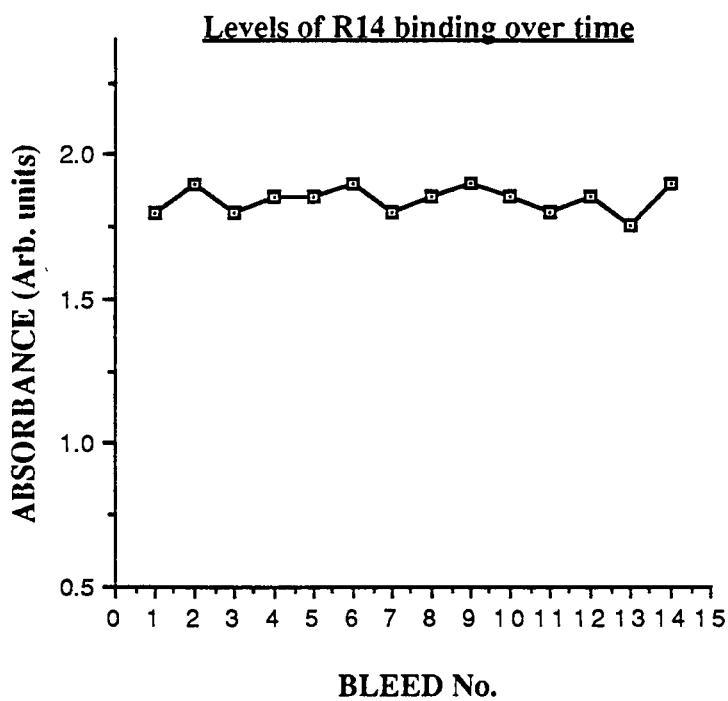


Figure 3iii) Results of ELISAs showing that the level of anti-PSD protein immunoreactivity remained high in serum taken from 14 bleeds of the same rabbit between 4/11/87 and 21/7/88.

Results.

The results of the immunoassay are presented in Figure 3iii). The assay revealed that the levels of antibody directed against PSD protein remained high in all serum samples from fourteen bleeds taken between 4/11/87 and 21/7/88.

Sera from all bleeds were pooled and an immunoglobulin-rich protein fraction was precipitated by the dropwise addition of an equal volume of saturated ammonium sulphate. The mixture was left for 30min at room temperature and the precipitate and supernatant separated by centrifugation at 15,000gmax. The supernatant was discarded and the precipitate dialysed at 4°C against two changes of PBS over 24hr. The protein content of the serum precipitate was determined and it was aliquotted and frozen prior to use. For immunoassays and Western blots, unless otherwise stated, whole serum refers to this dialysed precipitate at a protein concentration of 1mg/ml in PBS.

3vii) Titration of R14.

Using the ELISA procedure, the binding capacity of R14 was titrated by 1)keeping the antigen level constant and changing the antibody concentration 2)keeping the antibody concentration constant and changing the antigen concentration.

To find the most appropriate dilution of serum for use in further ELISAs the antiserum was titrated using the procedure detailed in the preceding section, all dilutions being made in washing buffer. Crude forebrain homogenate was used as antigen. To titrate the antibody, the homogenate was added to plates at a constant protein concentration of 10ug/ml and 50ul/well and antiserum was diluted serially tenfold giving solutions of neat

antiserum and 1:10, 1:100, 1:1000 and 1:10,000 dilutions.

To titrate antigen, homogenate was added to wells at protein concentrations diluted from 300µg/ml serially twofold down to 0.25µg/ml (50µl/well) and antiserum was used at a constant dilution of 1:1000.

Results.

The results are presented in Figures 3iv)A. (constant antigen - variable antibody) and 3iv)B. (constant antibody - variable antigen). The classic shape of the titration curves show that antibody-antigen binding is cumulative, that both antigen and antibody levels are limiting and that the binding is saturable. The results suggest that the most appropriate serum dilution is 1:500 - 1:1000 and the most appropriate antigen dilution is 5 - 10µg/ml of forebrain homogenate.

3viii) Western Blotting.

The molecular weights of the epitopes to which R14 antibodies bind were determined by the use of SDS-PAGE followed by Western Blotting (Towbin et al., 1979). We also included a brain tubulin preparation since this protein has been reported to be an antigenic determinant in antisera raised against PSDs (Nieto-Sampedro et al., 1981). 50µg/track each of crude homogenate, SPM protein, PSD protein and tubulin were electrophoresed on a 5% - 15% SDS polyacrylamide gel made using a resolving gel of pH 8.8 and a stacking gel of pH 6.8, containing appropriate concentrations of acrylamide with added ammonium persulphate and N,N,N'-N'- tetramethylethylenediamine (TEMED) to promote polymerisation. Gradient gels were poured using an LKB 2120 varioperpex II

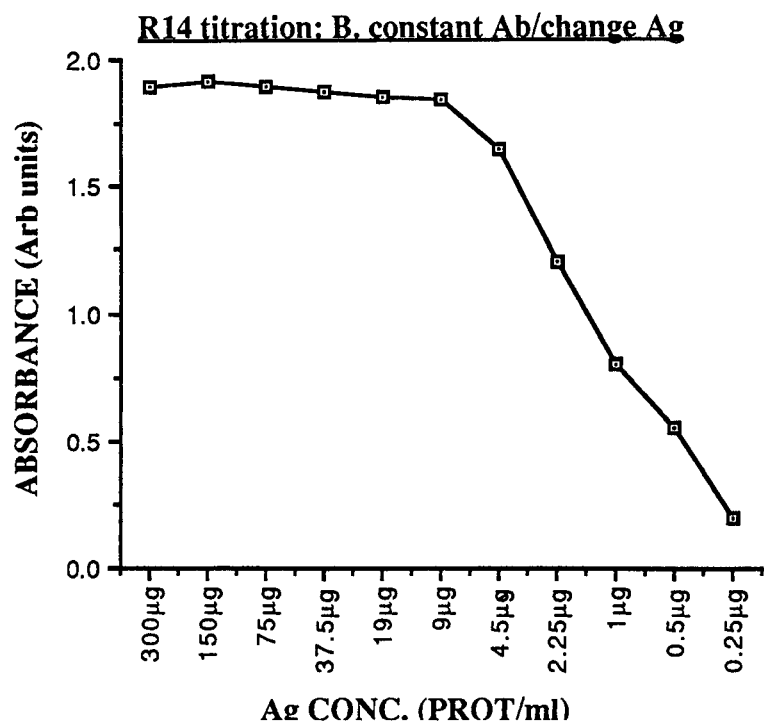
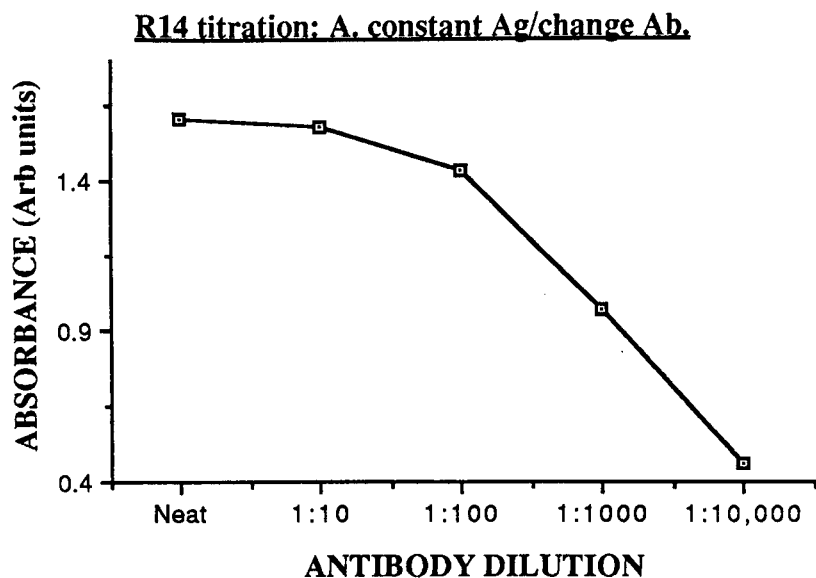


Figure 3iv) A. Amount of R14 antibody binding to crude homogenate of chick forebrain (50µl /well, 10µg protein/ml) - effect of changing antibody dilution. B. Amount of R14 antibody (diltution 1:1000) binding to crude homogenate - effect of changing antigen concentration.

peristaltic pump leading from a continuously mixing gradient forming apparatus. Molecular weight (MW) markers were included to determine the relative molecular weights of the electrophoresed proteins. Gels were run at a constant current of 25mA until the dye front migrated into the resolving gel whereupon the current was increased to 40mA until the dye reached the bottom of the gel. The gel was then removed from between the glass plates.

If the separated proteins were not to be transferred to nitrocellulose paper for probing with antibody, the gel was stained with 2% Coomassie brilliant blue in a solution containing 40% methanol, 10% acetic acid and 50% water then destained in a similar solution minus Coomassie until the background was clear.

If the separated proteins were to be probed with antibody, they were transferred to nitrocellulose paper according to the method of Towbin (1975). The gel was laid against a sheet of nitrocellulose and the gel/nitrocellulose sandwiched between two sheets of filter paper and then the whole complex sandwiched between two sheets of foam. This was held in place by a plastic support and the whole assembly was slotted into place in a blotting tank. Proteins were transferred from the gel to the nitrocellulose blot in electrophoresis buffer/20% methanol by passing a current of 200mA for 90min. The whole apparatus was cooled by circulating tap water.

The blot was removed and residual binding sites on the nitrocellulose were blocked by incubating with washing buffer - a modification of BLOTTO (Johnson et al., 1984) - (Tris-buffered saline, pH 7.4; 0.1% Tween 20; 5% milk powder) for 1 hr at room temperature. The nitrocellulose was washed X3 for 15min/wash in washing buffer. Each piece was incubated overnight with antibody at an appropriate dilution in washing buffer and washed X3. Nitrocellulose was incubated for 2hrs at room temperature with

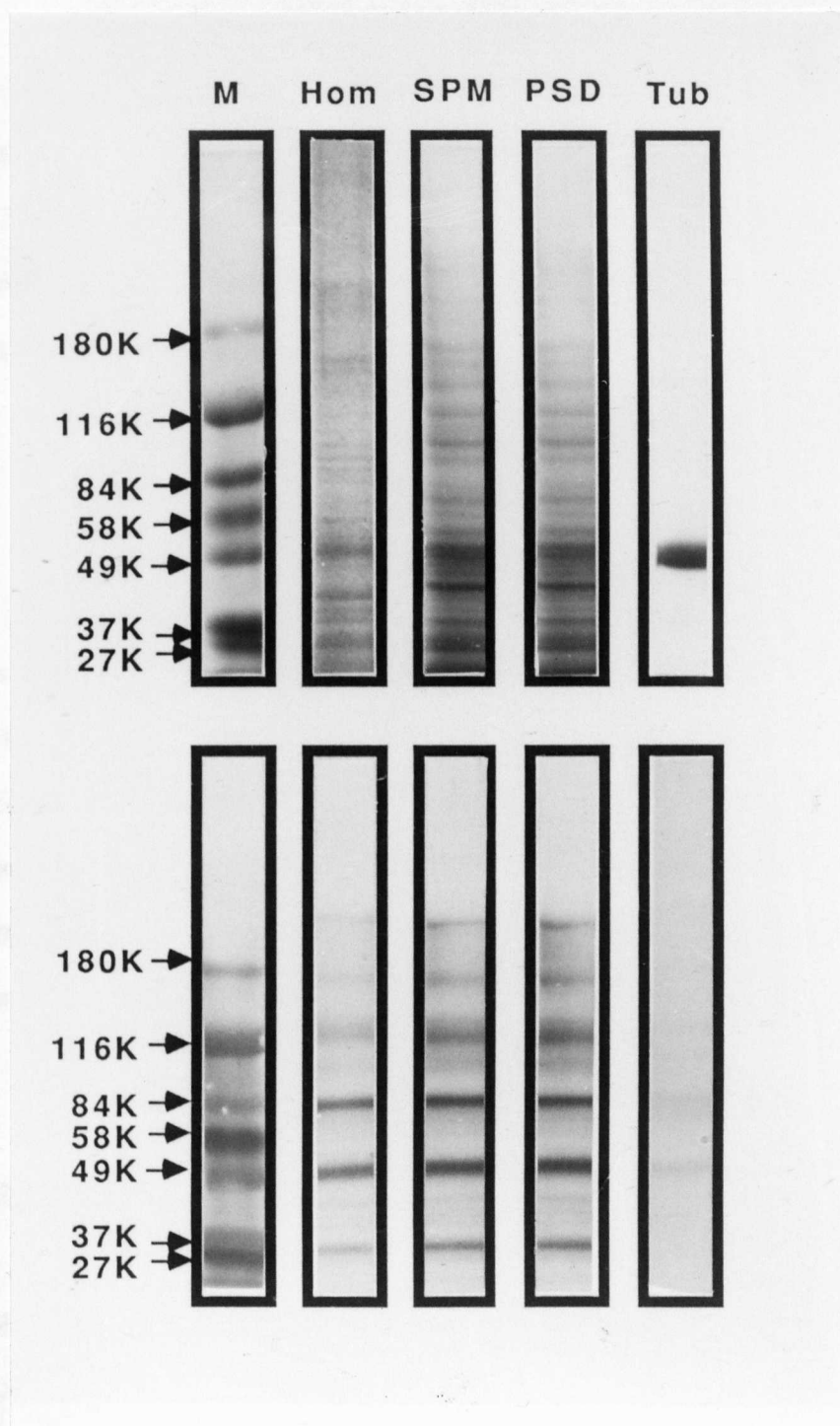


Figure 3v) SDS-PAGE (top panels) and corresponding Western blot probed with R14 (bottom panels). M = molecular weight markers, Hom = crude forebrain homogenate, SPM = synaptic plasma membrane protein, PSD = postsynaptic density protein, Tub = tubulin.

peroxidase-labelled F(Ab)₂ fractions of goat anti-rabbit IgG at a dilution of 1:500 in washing buffer, washed X3 in washing buffer and X1 in Tris-buffered saline/ 0.05% Tween 20. The antibody- binding protein was disclosed with 0.05% diaminobenzadine tetrahydrochloride, 0.01% H₂O₂ in TBS and the reaction quenched with distilled water when bands became visible.

Results.

The results of a typical SDS-PAGE and Western Blot are shown in Figure 3v). R14 recognises 6 major bands with molecular weights of approximately 230kDa, 170kDa, 135kDa, 90kDa, 55kDa and 30kDa. The exact identities of the antigens to which these proteins correspond are unknown, although they may be related to other known synaptic membrane antigens (see discussion). The Blot also shows that the antigen at 55kDa does not correspond to chick brain tubulin.

3ix) Subcellular Distribution of R14 Antigens.

ELISAs were performed on subcellular fractions obtained during the PSD preparation procedure (see section 3i)). Crude homogenate, S₂ (microsomes), P₃ (membranes), mitochondrial fraction, SPMs and PSDs of known protein concentration were allowed to bind to the microtest plates at concentrations of 10µg (± 2µg)/ml and 50µl/well. R14 was added at a dilution of 1:1000 and all other steps were as previously described in section 3v). Absorbances were divided by the protein concentration and each absorbance/[protein] measure was related to that for the crude homogenate.

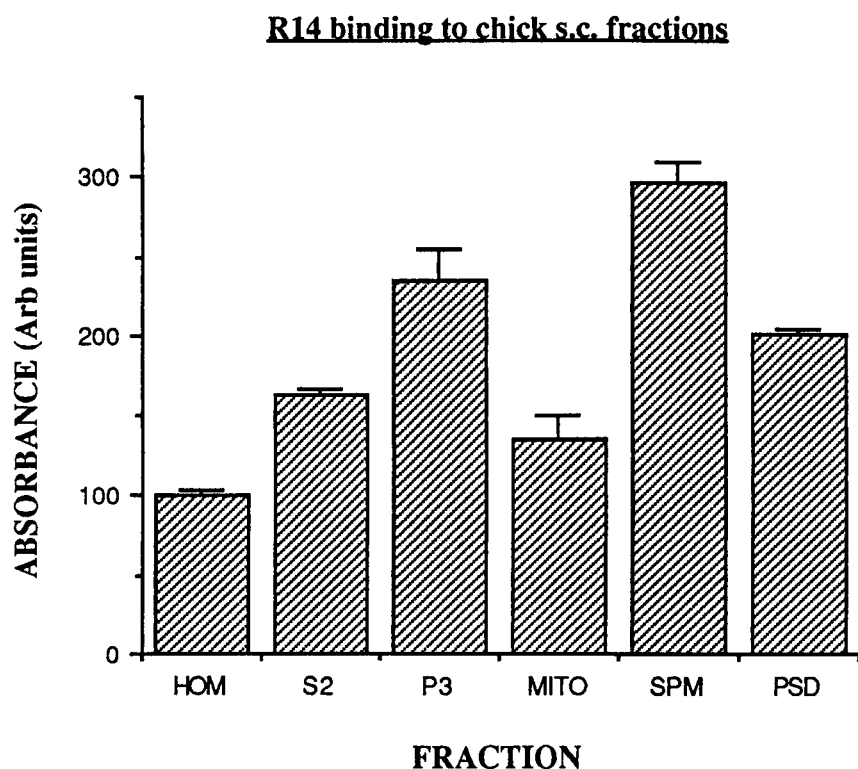


Figure 3vi) Relative levels of R14 immunoreactivity in various chick brain subcellular fractions. HOM = homogenate, S2 = microsomes, P3 = membranes, MITO = mitochondrial fraction; SPM = synaptic plasma membranes, PSD = postsynaptic densities.

Results.

The results of these experiments are presented in Figure 3vi). The results are consistent with a largely membrane-bound location for the antigens recognised by R14 because there is a relative elevation of antiserum binding to membrane-rich fractions compared with homogenate. The fractions which are particularly enriched are the P₃ (membrane) fraction (230% of homogenate binding) the SPM (synaptic plasma membrane) fraction (300%) and the PSD (postsynaptic density) fraction (200%). This indicates that the R14 antigens are membrane-located and that any synaptic enrichment is primarily pre- and postsynaptic rather than specifically postsynaptic.

DISCUSSION.

These results confirm that, when raising polyclonal antibodies to a heterogenous immunogen such as the PSD, few postsynaptic density antigens are specific to the PSD. However, it may be possible to raise monoclonal antibodies to PSD-specific antigens (see chapter 6).

Although we have not identified the proteins to which the R14 antigens correspond there are several potential candidates with similar molecular weights which have been reported to be immunogenic and contained within synaptic membrane fractions. The R14 antigens (molecular weights approximately 230kDa, 170kDa, 135kDa, 90kDa, 55kDa and 30kDa. These antigens are presented below although the author is aware that comparison made simply on the basis of similar molecular weight on one-dimensional gels is purely speculative.

The 230kDa antigen may correspond to fodrin (brain spectrin) or a related 240kDa glycoprotein enriched in cerebellar synaptic junctions and PSDs (Groswald and Kelly, 1984). Alternatively it may correspond to the 230kDa glycoprotein enriched in PSDs isolated using the octylglucoside method used here (Gurd et al., 1982; 1983) which is prevalent in forebrain PSDs but not those from the cerebellum, at least in the rat.

The 170kDa antigen may correspond to a glycoprotein of M.W. 175kDa identified as a postsynaptic antigen (Gordon-Weeks and Harding, 1983), the postsynaptic density glycoprotein, gp180 (e.g. Gurd, 1980;1988), or the PSD antigen recognised by our own monoclonal antibody, 411B (Bullock et al., 1987; 1988). Other possibilities for molecules related to this R14 antigen include the N-CAM family, having isoforms of M.W. 180kDa, 140kDa and 120kDa (Edelman, 1985; Rutishauser and Jessel, 1988).

R14 recognises an antigen of M.W. 135kDa which may share homology with one of the con A-binding proteins identified in PSDs with molecular weights of 130kDa and 145kDa (Gordon-Weeks and Harding, 1983). Alternatively, it may correspond to the developmentally-regulated sialoglycoprotein of M.W. 130kDa recognised by the monoclonal antibody, F3-37-8 (Lakin and Fabre, 1981; Lakin et al., 1983). A brain specific antigen located, in part, at synapses but also found in white matter and myelin fractions (Gordon-Weeks et al., 1989). The antigen may also be related to a cytoskeletal-associated membrane glycoprotein identified in chick brain of M.W. 130kDa and reported to be concentrated at synapses (Ranscht et al., 1984).

Proteins of similar molecular weights to that of the 90kDa antigen recognised by R14 have been reported. A non-con A-binding polypeptide of 85kDa is present in isolated PSDs (Gordon-Weeks and Harding, 1983) and a protein of M.W. 95kDa, termed PSD-

90, was one of three major antigens recognised by antibodies to bovine PSDs (Nieto-Sampedro et al., 1981).

We have established that the major 55kDa antigen recognised by R14 is not tubulin (see above). Proteins which may share homology with this antigen include the 50kDa mPSDp (e.g. Gurd, 1980; 1988), the antigenic, synaptically-enriched gp50 (Beesley et al, 1986; 1987). The protein may be related to the antigenic glycoprotein which resolves on SDS-PAGE into a diffuse band at 55kDa, gp55, although this antigen appears to be absent from PSDs (Hill et al., 1987; 1988).

The 30kDa band observed on Western Blots may be similar to the low molecular weight acidic glycoproteins, the ependymins (e.g. Shashoua, 1989). Alternatively, it may be related to a PSD protein of 29kDa identified by Gordon-Weeks and Harding (1983). It is not clear from the data they present whether this protein is glycosylated.

Although the antigens recognised by R14 appear to have a membrane origin, we cannot assume that all, or any, of the antigens are glycoproteins. This could, however be addressed in at least two ways. If the R14 antigens are glycoprotein in nature then the epitopes which are recognised are likely to reside on the polypeptide rather than the carbohydrate structure. Cleaving the carbohydrate with an appropriate endoglycoside would therefore produce a band shift as observed by immunoblots. Alternatively, one could incubate tissue with radioactive isotopes of putative sugar subunits, if the antigens are glycosylated then the isotopes would become incorporated into the molecule and immunoprecipitation procedures would reveal radioactivity within the antibody-antigen complexes.

CHAPTER 4: EFFECT OF INTRACRANIAL INJECTIONS OF ANTIBODY R14 ON PASSIVE AVOIDANCE LEARNING IN THE CHICK.

INTRODUCTION.

Many studies using immunological techniques to delineate molecular changes occurring during learning and memory formation have concentrated on the potential of certain antibodies (both polyclonal and monoclonal) to inhibit specific forms of learning. Examples of such studies are discussed in the following section.

An early investigation by MacPherson and Shek (1970) found that immunisation with crude microsomal fractions from rat brain or liver resulted in the production of iso-antibodies. Immunised rats demonstrated impaired learning on a visual discrimination, active avoidance task. However the results of this study are hard to interpret since iso-antibodies to the same fraction from liver had a similar effect. The study does demonstrate, however, that antibodies are capable of impairing learning.

Following from studies which found that intraventricular infusion of anti-Synaptic Membrane Fraction (anti-SMF) resulted in the production of non-convulsive epileptiform EEG (Proctor-Bowen et al., 1971; Karpiak et al., 1973), Karpiak & Rapport (1975) examined the effects of prenatal exposure to anti-SMF antibodies on subsequent learning. Pregnant rats were injected with anti-SMF and their offspring were exposed to learning on a differential reinforcement task. Antibody-treated animals displayed profound deficiencies on the learning of the task although it is not clear whether prenatal exposure to antibodies resulted in an inhibition of development which manifests itself as a learning

deficit. Karpiak and co-workers did go on to demonstrate a more direct effect of antisera on learning in a series of studies in the late 1970s. These are discussed below.

The potential of using antisera to disrupt both EEG and learning was examined by Karpiak et al (1976). They studied the effects of intraventricular infusion of antibodies to a synaptic membrane fraction (SMF) and to the S-100 protein. Both antibodies were administered after training on a maze-learning task, and again after testing on day 2. Amnesia, measured as increased latency to reach a performance criterion, developed over days 2 to 5 in both antibody-treated groups, the mean latency being approximately double that of controls. With respect to the EEG experiment, anti-SMF resulted in a epileptiform EEG with no discernable effects of anti-S-100. This suggests that the amnesic effect of the anti-SMF serum on maze-learning may not be a direct result of the epileptiform electrical activity.

The effect of antiserum to the ganglioside G_{M1} on learning in the rat has been investigated (Karpiak et al., 1978). Intraventricular infusion of anti- G_{M1} was performed immediately following training on a step-through avoidance paradigm. On testing one week later antibody treated animals showed only 10% recall compared to a group of animals treated with antibody-absorbed serum.

A related early study found an amnesic action of antibodies to G_{M1} , on passive-avoidance learning in the mouse (Karpiak & Rapport, 1979). This effect was very unspecific, causing amnesia when administered up to 5hrs before or 19hrs after training and there was also inhibition of retrieval when the antiserum was administered 24hrs or 48hrs after training.

Kobiler et al (1976) examined the effect of infusion of anti-synaptic plasma membrane (SPM) antibodies on a variety of tasks. 2ml of an immunoglobulin-rich fraction of anti-SPM serum was administered by intraventricular infusion. Antibody-treated groups displayed 20 - 50% recall compared to an absorbed antibody control group in a conditioned emotional response paradigm. In a spatial task the anti-SPM group the antibody group took 2.5 times longer to find a reinforcer than controls. In a step-down passive avoidance paradigm, the latency in the antibody group was 27% and 46% that of controls at 24hrs and 48hrs respectively and, in a black/white discrimination task, animals which received an infusion of anti-SPM antibodies made four times the number of errors made by controls. Although this study shows that administration of anti-neural antibodies are capable of disrupting a variety of tasks, the time-course of the amnesic action is unclear since, in the various tasks the antiserum was administered 1hr before or 2hrs, 24hrs and 72hrs following training.

Antisera raised to glycoproteins have been shown to have effects on learning and memory in a variety of tasks. One important body of work which has relied heavily on the use of antibody technology to study learning and memory is the study of the ependymins. The goldfish ependymins β and γ are low molecular weight acidic glycoproteins secreted by a group of around 15,000 non-neuronal cells. They are hypothesised to be involved in increasing synaptic efficacy by release into the synaptic cleft and subsequent polymerisation at the synapse in response to local changes in calcium ion concentration, once in the extracellular space they are postulated to form a matrix for cell growth (Shashoua, 1985; 1990).

Anti-ependymin antiserum administered to goldfish by intraventricular injection was shown to inhibit the acquisition of a vestibulomotoric training task (Shashoua & Moore, 1978) although whether the task, in which goldfish compensate for the effect of an

attached buoy, can be described as "learning" is debatable. Similar administration of antibody injected 8 or 20hrs following training in an active shock-avoidance task reduced retention to 50% the level of non-injected animals (Shashoua, 1985), results which were replicated by Piront & Schmidt (1988).

These studies are difficult to interpret since the time window of amnesic effect (antibody administration any time between 0.5 and 24 hrs post-training) is much longer than that observed in other studies of protein and glycoprotein synthesis inhibition although it may reflect the inhibition of a long-term ongoing consolidation period. Immunoassays for ependymins in extracellular fractions have revealed training related changes in their levels following the vestibulomotor task (Schmidt, 1987) and following classical conditioning (Shashoua and Hesse, 1989). Isolated ependymin can be induced to polymerise in vitro by reducing calcium to sub-physiological levels (Shashoua, 1985) but it has yet to be demonstrated that incubation with anti-ependymin antibody inhibits this polymerisation.

Further work on the amnesic effect of anti-brain glycoproteins has been undertaken by Regan's group in Dublin (Nolan et al., 1987a; 1987b). F-3-87-8, a monoclonal antibody which recognises a developmentally regulated sialoglycoprotein of M.W. 130kDa, was administered by intraventricular infusion 5min before training on a rat passive avoidance paradigm. Antibody-treated animals displayed total amnesia for the task when tested 24hrs or 48hrs following training as defined by failure to reach a time criterion of 3mins on a safe platform. The antibody appeared to result in an acquisition deficit since it was not effective when infused 6hrs or 10hrs post-training.

The same task was used to examine the amnesic potential of antibodies to the brain specific protein 2 (BPM) and D2-CAM/N-CAM (Nolan et al., 1987b). Anti-BPM, administered 5min before the onset of training, resulted in amnesia in animals tested 24hrs

or 48hrs post-training (18% recall at both test times) compared to controls which included animals receiving antibody-absorbed serum and sham-operations. No amnesia resulted when anti-BPM was administered 6hrs or 10hrs post-training. In this study the authors found no amnesic effect of anti-D2-CAM/N-CAM infused 5min before training however, further studies revealed that antibodies to N-CAM are capable of disrupting learning when administered within a time-window 6hrs to 8hrs post-training, but not at other times, and amnesia was apparent only at the 48hr test time (Doyle et al., 1990). The authors also reported that antibodies to the putative cell adhesion molecule, amyloid precursor protein (APP) were amnesic, when administered immediately before training or 2.5hrs following it, at both test times. These studies indicate the potential of antibody technology in dissecting the complex temporal relations between cell-surface molecules involved in establishment of a memory trace and lend support to the hypothesis that there is a second wave of glycoprotein synthesis which is involved in consolidation of long-term memory.

Antiserum to the neuropeptide hormone vasopressin, administered by intraventricular injection, immediately following a one-trial passive avoidance task in the rat resulted in an almost complete loss of retention at 6, 8, 24 or 48hrs post-training. There was no deficit in rats tested 2min, 1hr or 2hrs following training suggesting that the antiserum was causing a consolidation deficit, although the time of administration of the antiserum was not changed to in this study. Intravenous administration of 100 times as much antibody had no observable effect on learning (van Vimersa Greidmas et al., 1975a).

Daily intraventricular administration of anti-vasopressin 30min prior to a pole jumping avoidance task resulted in a slowing of learning and extinction in the antiserum group was faster than in controls (van Vimersa Greidmas et al., 1975b). Similar results have been obtained on a passive avoidance task (Croisier et al., 1990) although the role of vasopressin in central memory processes is unclear - the neuropeptide is involved in

bradycardiac responses (Bodus, 1975) suggesting that effects on learned passive avoidance may result from increased parasympathetic behaviour.

Both the production and maintenance of long-term potentiation (LTP), can be blocked with monoclonal antibodies raised against 5 day-old rat dentate gyrus dentate gyrus (Stanton et al, 1987). The monoclonal antibody, B6E11 (which recognises an epitope of M.W. 42kDa) blocked production of in vitro slice LTP and suppressed established LTP when applied locally to apical dendrites synapsing with the potentiating input to the dentate gyrus. There was no effect of the antibody when applied to either basal dendrites or cell bodies and a different monoclonal antibody generated from the same panel and with similar immunohistochemical properties did not impair LTP. This study illustrates the power of using antibodies to produce very local effects.

The effect of antibodies on passive avoidance learning in the chick has been studied using polyclonal and monoclonal antibodies to the Thy-1 glycoprotein (Bernard et al., 1983; Lappuke et al., 1987). A polyclonal anti-Thy-1 was amnesic for passive avoidance, when administered by freehand intracranial injection, between 5min pre-training and 10mins post-training when chicks were tested at 3hrs or 24hrs following the training presentation (Bernard et al., 1983). The course of amnesia was found to follow similar temporal parameters as that for protein synthesis inhibitors, with amnesia developing rapidly between 50 and 60min post-training. Control antisera (anti-rat Thy-1 and anti-chick cerebellum) had no apparent effect on memory retention. The study was followed up using a monoclonal anti-Thy-1 (Lappuke et al., 1987) and again the antibody was amnesic, when administered by intracranial injection, within a time window 5min before to 5min post-training. The same antibody resulted in amnesia when injected subcutaneously into a fold of skin in the ventral side of the rib cage 5min pre-training. However, amnesia was not apparent when the monoclonal was injected subcutaneously

30min pretraining. It seems unlikely that there could be a large difference in the amount of antibody around target brain tissues between the two time points when administered via the subcutaneous route. Perhaps the way to resolve this apparent paradox would be to probe the brain for antibody using a labelled anti-mouse IgG at 5 and 25min following subcutaneous injection

The aim of the following experiments was to establish whether R14 recognises, and is capable of inhibiting the function of, antigenic proteins which play a role in learning and memory formation in the young chick. Further experiments were directed at defining the temporal parameters of any resulting amnesia and at localising antibodies following their intracranial injection.

METHODS

4i) Affinity Purification of R14 IgG.

To ensure that any behavioural effect of intracranial injections of R14 could be attributed to binding of antigen species observed by Western Blotting (and not to binding by other, non-IgG immunoglobulins or non-specific effects of serum proteins) it was important to isolate IgG from whole serum for use in behavioural pharmacology experiments. This was achieved by affinity chromatography using Protein G sepharose (Pharmacia, UK).

Any particulate matter remaining in the R14 serum precipitate (12mg/ml protein) was cleared by centrifugation at 10,000rpm followed by filtration through a .22 μ m filter. The pH was adjusted to 7.0 (\pm 0.2) by the addition of 100mM sodium phosphate, pH 7.0. The serum was applied to swollen Protein G Sepharose which had been washed extensively with 20mM phosphate, pH 7.0. All material running through the column was collected in

20 drop (~1min) fractions. The column was washed for approximately one hour in 20mM phosphate, pH 7.0, after which IgG was eluted with 0.1M glycine/HCl, pH 2.7, and the eluate immediately taken to neutral pH by the addition of 55µl/fraction of 1M Tris/HCl, pH 9.0.

Relative protein values were determined using a modification of Bradford's method adapted for 96-well microtitre plates. Fractions making up the the second peak to be eluted from the column (IgG) were pooled and dialysed against 2 changes of 2 litres saline over 36hrs. The resulting purified IgG was characterised using SDS-PAGE, the protein concentration adjusted to 1mg/ml, aliquoted and frozen prior to use.

Results.

The relative protein content of the fractions coming off the protein G sepharose column in a typical preparation are shown in the top panel of Figure 4i). The first peak is the unbound material (non-IgG protein) and the second is the IgG eluate. Purity of the fractions was determined by SDS-PAGE (see Section 3vii)) of equal amounts of starting material, the unbound fraction and the IgG fraction (Figure 4i), bottom panel). Bands corresponding to the reduced heavy (H) and light (L) chains of IgG (M.W. ~50kDa and ~25kDa respectively) are present (along with other bands) in the starting fraction, are absent in the unbound fraction and are the only, highly concentrated, bands in the IgG eluate. This demonstrated the purity of the isolated IgG fraction.

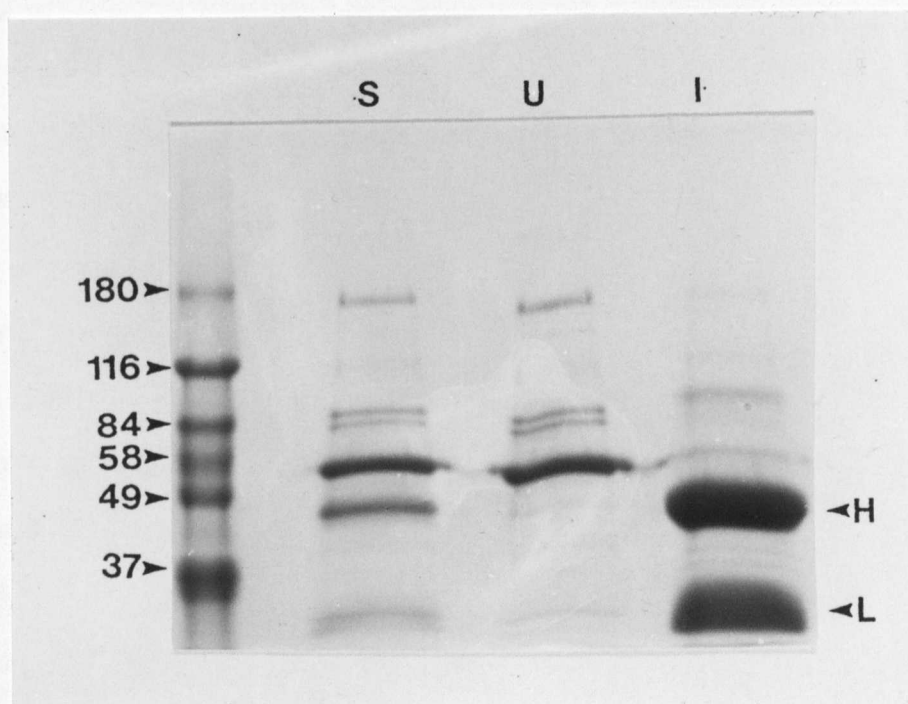
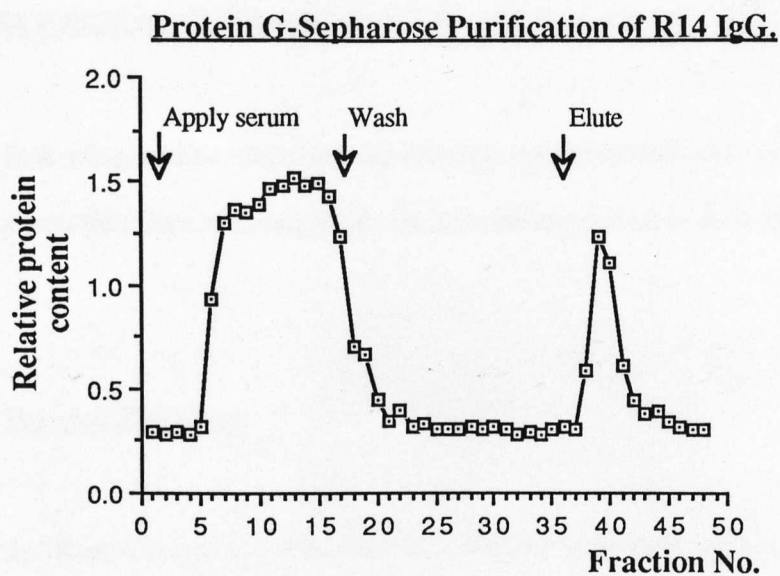


Figure 4i) Protein G sepharose purification of R14 IgG. Top figure, relative amount of protein in fractions running through a protein G sepharose column. Bottom figure, SDS-PAGE of samples of pooled fractions. Track 1 = molecular weight markers, S = whole serum precipitate, U = unbound material, I = IgG fraction.

BEHAVIOURAL PHARMACOLOGY.

The following section describes experiments aimed at defining the temporal parameters of any amnesic effect resulting from intracranial injections of R14 IgG.

4ii) Training Procedure.

Fertile "Ross 1" eggs were hatched in a communal brooder maintained at 38 - 40°C on an alternate 12hr light/dark cycle. 24 - 36hrs post-hatch chicks of both sexes were placed in pairs into open-topped aluminium pens (length 25cm, width 20cm, height 20cm) with each pen illuminated from above by a 25W red bulb. Animals had free access to scattered chick crumb and were left in the pens for 30 to 60 mins to equilibrate with the environment prior to any training.

Chicks were trained essentially as described previously (Rose et al, 1980). Each bird received 3 pretraining trials separated by 5 minute intervals, each consisting of a 10 second presentation of a dry, white bead (diameter 2.5mm). Only birds pecking at the bead on at least two pretraining trials were used in the experiment. Thirty minutes after the start of pretraining each bird was given a training trial which consisted of a 10 second presentation with a chrome bead (diameter 4mm) coated with bitter-tasting methyl anthranilate (MeA). Pecking at the MeA bead elicits a stereotyped "disgust response" - the animal shakes its head, backs away from the bead and wipes its beak on the floor of the pen (Gillan, 1979). On subsequent presentations of a similar but dry chrome bead, MeA-trained birds will avoid the bead for up to 48 hours post-training.

24 hrs after training the animals were tested for retention by a 10 second presentation of a dry chrome bead similar to that used in the training trials. A simple binary measure was employed at test in which chicks were scored as "peck" or "avoid".

4iii) Effect of Changing the Time of Injection of R14 IgG Relative to Training.

The aim of this experiment was to determine if intracranial injection of R14 IgG purified according to the previously-described method was capable of causing amnesia in chicks trained on a passive avoidance task, and if so, at which time points relative to training it had this effect.

IgG isolated from R14 (protein concentration = 1mg/ml in saline) was administered by bilateral intracranial injection of 10 μ l/hemisphere. Injections were performed 60min, 30min or 15min pre-training or 10min post-training. The depth of penetration and the site of injection (the IMHV) were controlled using a sleeved Hamilton syringe and a special head holder respectively. In this experiment control animals received bilateral intracranial injections (10 μ l/ hemisphere) of saline alone. The injections were performed very quickly and caused no apparent distress to the animals.

Results.

Under normal circumstances (i.e. without pharmacological intervention), using the training protocol described above at least 70% - 80% of M birds will avoid the bead on test. M birds which peck the bead on test are considered to be amnesic.

Treatment	Time of injection relative to training			
	-60min	-30min	-15min	+10min
Saline	81% (16)	93% (15)	60% (15)	80% (15)
R14 IgG	50% (16)*	75% (16)	42% (12)	57% (14)

Table 4i) Percentage avoidance following bilateral intracranial injections of saline or R14 IgG administered 60min, 30min or 15min pre-training or 10min post-training. All birds were MeA-trained and tested 24hrs post-training. Numbers per group are presented in parentheses. (*; $p \leq 0.02$ compared with controls as determined by χ^2).

The results of the experiments using the antiserum are presented in table 4i) and represented graphically in figure 4ii). Bilateral injections of R14 IgG caused amnesia for the passive-avoidance task in a significant number of animals injected 60min pre-training and tested 24hrs post-training, with 50% of antibody-treated animals avoiding compared to 81% of saline-injected controls ($\chi^2 = 5.87$, $p < 0.02$). If the time of injection was changed to 30min or 15min pretraining or 10min post-training the antibody-treated group did not differ significantly from saline-treated controls. In all cases N was between 12 and 16. The reduction in the percentage retention of both groups of birds at the -15min time point is a phenomenon often observed in pharmacological studies when agents are injected at this time point (Patterson, personal communication). The reason for this is unknown but it may reflect the coincidence of training with some phasic response to the impact of injection.

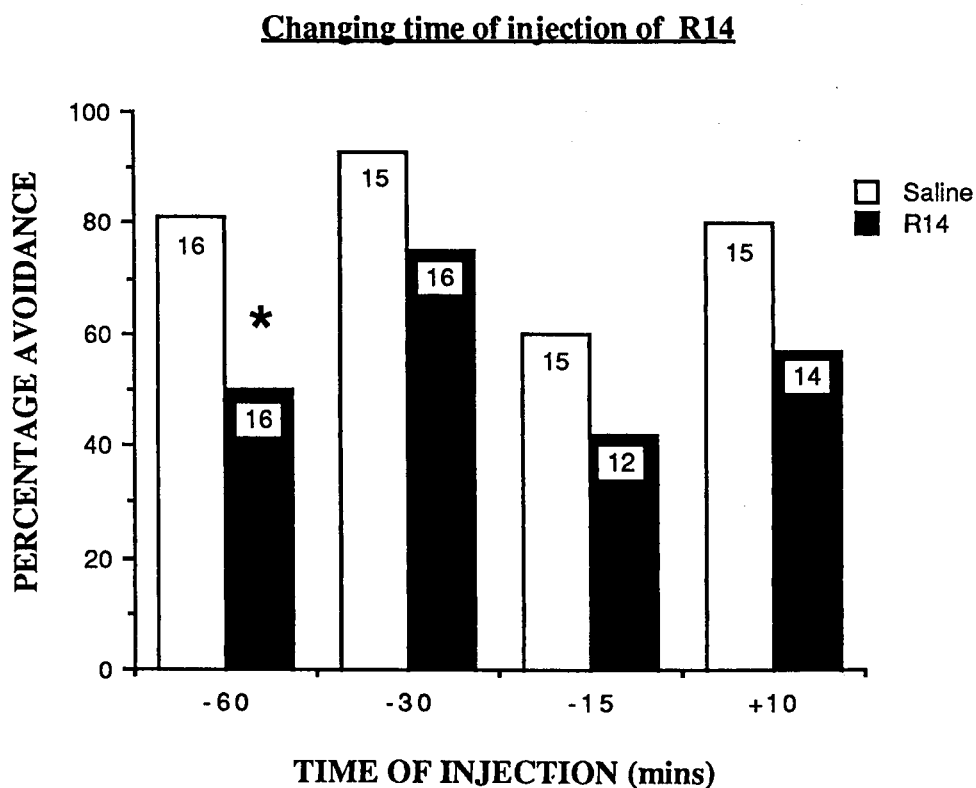


Figure 4ii) Retention for the passive avoidance task as expressed as percentage of M-trained animals avoiding a bead on test 24hrs post-training in groups of animals receiving bilateral intracranial injections of R14 IgG administered 60min, 30min, 15min pre-training or 10min post-training. Inserts in the bars are numbers of animals per group (*, $P < 0.02$ as determined by χ^2).

4iv) Changing the Time of Test.

This experiment was aimed at determining if the antibody-induced amnesia observed in the previous section could be observed in animals tested at time-points earlier than 24hrs following training. This time, as well as saline injected controls, additional control groups were included which received intracranial injections of IgG purified from serum obtained from the same rabbit which provided the R14 antiserum, but before the immunisation regime began (pre-immune rabbit serum, PIRS). This was to test for the possibility of non-immunogen related antibodies having a task-related behavioural effect on the chicks.

Nine groups of animals were used; three groups injected with saline 60min pre-training and tested at either 1hr, 3hrs or 24hrs post-training and a corresponding three groups each allocated to a pre-immune IgG (PIRS) group and to a R14 IgG-injected group (N = 19 - 21 in each group).

Injections and Training.

All injections were administered 60min pre-training by bilateral intracranial injection of 10µl/hemisphere (1mg/ml in saline for both PIRS and R14), exactly as described in the preceding section. The housing and training procedures were as previously described except that animals were tested at either 1hr, 3hr or 24hrs post-training, depending on the group to which they had been assigned.

Results.

Treatment	Time of test post-training		
	1hr	3hr	24hr
Saline	75% (20)	80% (20)	85% (20)
PIRS	68% (19)	81% (21)	79% (19)
R14 IgG	60% (20)	57% (21)	48% (21)*

Table 4ii) Percentage avoidance following bilateral intracranial injections of saline, pre-immune serum IgG or R14 IgG administered 60min pre-training. All birds were MeA-trained and tested 1hr, 3hrs or 24 hrs post-training with numbers per group presented in parentheses. (*; $p \leq 0.02$ compared with saline-treated group and $p \leq 0.05$ compared with the PIRS group as determined by χ^2).

Intracranial injection of R14 IgG was amnesic, when administered 60min pretraining, only in the group of animals tested at the 24hrs post-training time point (Figure 4iii); Table 4ii)). This difference was statistically significant when the R14 group, with 48% retention, was compared with the saline-treated control group with 85% retention ($\chi^2 = 6.438$, $p < 0.02$) and when compared with the group injected with pre-immune serum IgG which showed 79% retention ($\chi^2 = 4.177$, $p < 0.05$). Although there was a trend for

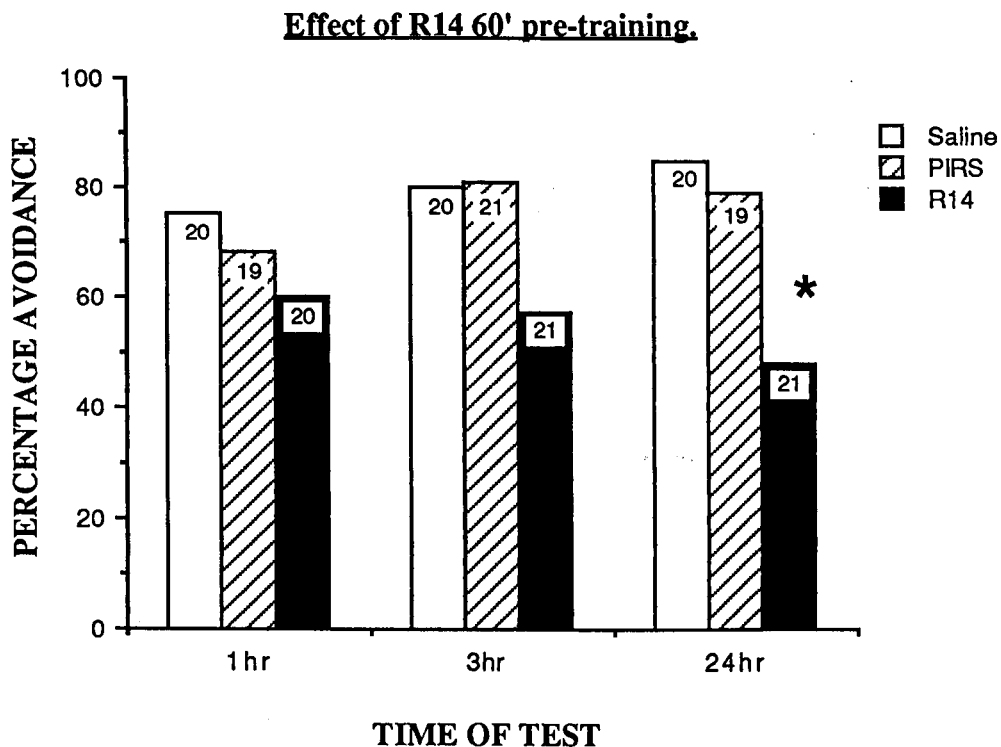


Figure 4iii) Retention for the passive avoidance task as expressed as percentage of M-trained animals avoiding a bead on test in groups of animals receiving bilateral intracranial injections of R14 IgG, pre-immune serum IgG (PIRS) or saline administered 60min pre-training. Tests were performed 1hr, 3hrs or 24hrs post-training. Inserts in the bars are numbers of animals per group (*, $P < 0.02$ compared to saline group and $p < 0.05$ compared to PIRS group as determined by χ^2).

the R14-treated to show less recall than controls, there were no significant differences between R14-treated animals and either control group when the chicks were tested at 1hr or 3hrs post-training. It is possible, however, that the antiserum is resulting in the development of amnesia from the onset of training but that the differences between the control and R14 groups only reach the threshold of significance by 24hrs post-training.

4v) Effect of R14 on Chicks Trained on a Water-coated Bead.

In the previous two experiments we could not rule out the possibility of R14 having its effect on the experimental animals by increasing pecking behaviour in a non-specific manner not related to memory formation. This possibility was tested using three groups of animals; a saline-injected group trained on a bead coated with water rather than with MeA, a group injected with R14 IgG and trained on a water coated bead and an R14-injected group trained on the MeA bead, as in the previous experiments. Under normal circumstances, on subsequent presentations the water-trained (W) birds continue to peck at a similar, dry bead to the one used in the training trial and are usually used as controls in biological assays of chick brains following passive avoidance learning.

Injections and Training.

The injection protocol was identical to that described in the previous section. Animals received bilateral intracranial injections of either saline or R14 IgG 1hr before the training trial. Pretraining trials were exactly as described in the first experiment. Training consisted of the presentation of the chrome bead coated with either the aversive MeA (M birds) or with a similar bead coated with water (W birds), depending on the group to

which the animal had been allocated (N = 13 - 15). Half the R14-injected birds were trained on the M bead and half on the W bead. In this experiment all the saline-injected birds were trained on the W bead. Animals were tested 24hrs following training.

Results.

Treatment/ training	Percentage pecking
Saline	77% (13)
R14 /W	69% (13)
R14/M	53% (15)

Table 4iii) Percentage pecking following bilateral intracranial injections of saline or R14 IgG administered 60min pre-training. W = water-trained M = MeA-trained. Numbers per group are presented in parentheses.

The number of water-trained birds injected with R14 IgG and continuing to peck at the bead on test (69%) did not differ significantly from those injected with saline (77%) (Table 4iii)). This suggests that the amnesia observed in the previous experiments could not be attributed to a non-specific increase in pecking activity. In addition, the group of R14-injected birds trained on the water bead was statistically indistinguishable from the group receiving the same injection but trained on the MeA bird (53% avoidance).

This data reinforced the results of the previous experiments, confirming that pre-training bilateral injections of R14 IgG reliably result in amnesia in around 50% of birds tested at 24hrs post-training.

4vi) Localisation of Antibody Following intracranial Injection.

Many experiments from our laboratory have indicated that the forebrain areas IMHV, LPO and possibly the PA are the loci involved in the biological changes responsible for memory formation in the chick (see chapter 2). In this experiment we wanted to determine which of these areas contained detectable levels of antibody following the intracranial injections described above.

One hour following bilateral, intracranial injections of 10 μ l/hemisphere R14 IgG or of saline (i.e. the point of training in the previous experiments), chicks were killed. The brains were removed immediately, placed on ice and six brain regions were dissected out using a specially designed Araldite brain mould (see Figure 4v)) according to the following procedure. Regions corresponding to the left and right IMHV, LPO and PA were dissected out from coronal slices taken through the brain using razor blades whose position and angle of incision was determined by the position of slots cut through the mould. To ensure that the dissection was an accurate representation of the respective locus, pairs of each region were pooled. Each region pair was homogenised and the protein content was determined using a modification of Bradford's method (Bradford, 1976). A modification of the ELISA method employed in chapter 3 (see section 3iv)) was used to probe for IgG. 50 μ l of homogenate at 100 μ g/ml was added to each well following priming, the homogenate was allowed to bind overnight at 4 $^{\circ}$ C, plates were washed X5 and peroxidase-labelled goat anti-rabbit IgG antibody was added at a dilution of 1:1000. This and all subsequent steps were identical to that previously described in section 3iv).

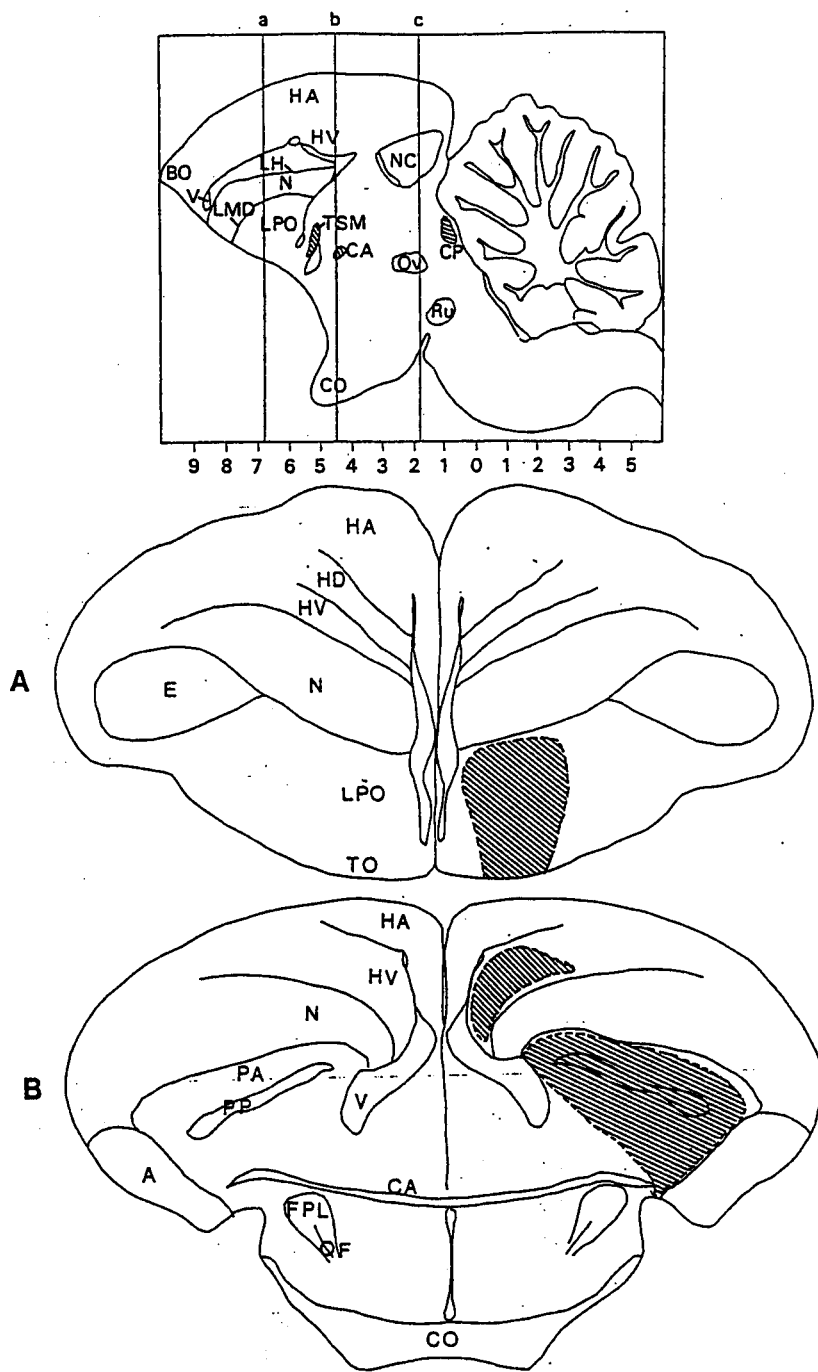


Figure 4iv) Schematic representation of coronal slices taken using a brain mould. Slice A was used for dissection of the LPO and slice B for dissection of the IMHV and PA. Areas corresponding to these loci are indicated by stippling (from Bullock et al., 1987) .

Results.

One hour following bilateral intracranial injection of R14 IgG, i.e. the point corresponding to training in amnesic chicks, antibody was detectable only in homogenates of the left and right IMHV. In the other forebrain regions examined (the left and right PA and LPO) the absorbance following ELISA did not differ from the background level measured in the saline injected controls (see Figure 4v))

4vii) Effect of R14 on Number of Pre-training and Training Pecks.

The effect of intracranial injection on generalised motor activity, as expressed by pecking, was examined by comparing the total number of pretraining and training pecks obtained from the experiments reported in this chapter in animals injected 60min pretraining (the time-point which resulted in amnesia in antibody-treated animals). Since this was the behavioural measure taken as an indication of amnesia at test it was considered to be a legitimate measure of non-specific motor activity which may confound the results of behavioural studies.

Massing the data from these experiments revealed that animals injected with saline 60min pretraining pecked on 92% of presentations (328/356), R14 IgG-injected animals on 91% of presentations (382/424) and PIRS IgG-treated animals on 89% of presentations (210/236). This indicates that the antibody had no effect on mere pecking activity but that the increases in pecking on test were a direct consequence of amnesia for the passive avoidance task.

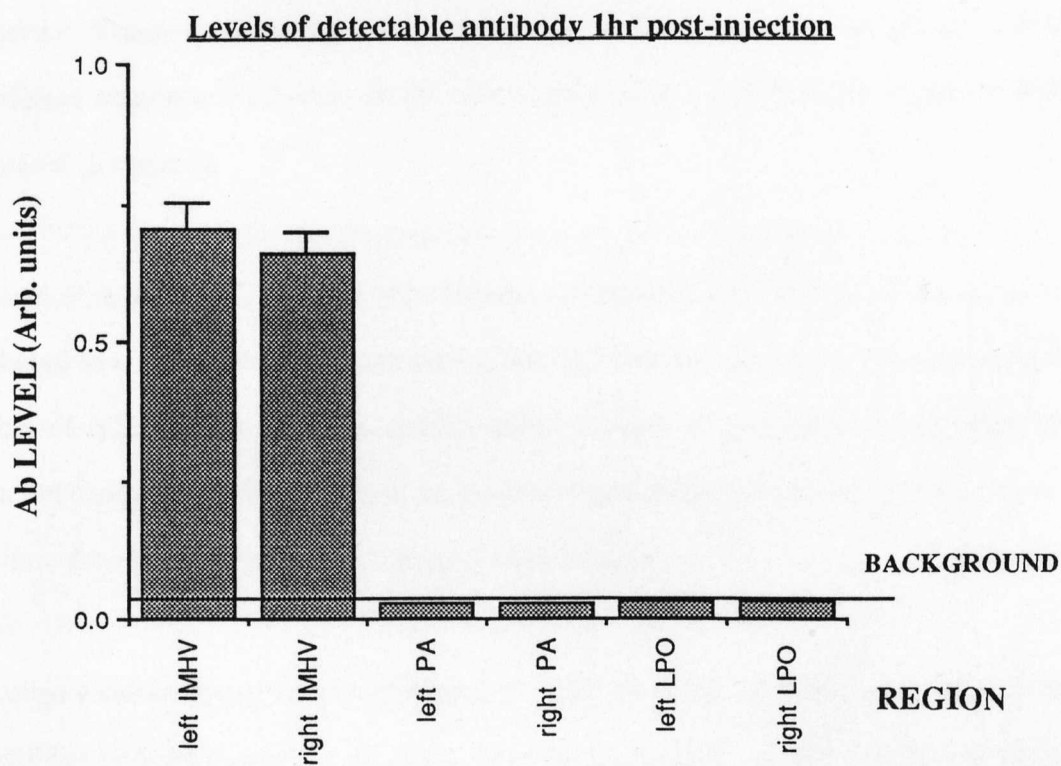


Figure 4v) Relative levels of detectable antibody in the IMHV, LPO and PA one hour following intracranial injection of R14 IgG. Control level refers to the mean absorbance in the same loci taken from saline injected animals.

DISCUSSION.

IgG from R14 serum has been shown to act as an amnestic agent in the chick. Bilateral intracranial injections of R14 IgG (1mg/ml; 10 μ l/hemisphere) reliably result in amnesia in approximately 50% of chicks if antiserum is injected 60min prior to training and chicks are tested 24hrs post-training. If the time of antibody injection is 30min or 15min pre-training or 10 min post-training no amnesia occurs. Similarly, chicks injected with antiserum 60min pre-training and tested at 1hr or 3hrs post-training do not exhibit significant amnesia. Although, in all cases, there is less recall in R14-injected birds compared to controls.

The use of isolated IgG in these experiments ensures that the amnesic effect cannot be attributed to antigen binding by unknown, non-IgG immunoglobulins or to non-specific effects of other serum proteins. Additionally, the use of controls injected with IgG prepared from pre-immune serum as a control precludes the possibility of amnesia resulting from unknown, non-immunogen related IgGs.

Although a thorough test battery of behavioural effects of the antiserum was not used, the possibility of a non-specific, antibody-induced increase in pecking behaviour being manifested as amnesia can be excluded. Animals injected with R14 IgG and trained on a water bead displayed similar amounts of pecking to a saline injected group and there was no apparent effect of R14 on total number of pecks in pre-training and training trials.

The antiserum remains localised to the site of injection (i.e. the IMHV) for at least 60min following injection. This probably reflects the relatively large size of the molecule (M.W. of IgG ~ 150kDa) which was injected into the relatively dense tissue of the IMHV. Other data have suggested that immunoglobulins do not diffuse through the chick brain to the

same extent as many smaller molecules used for pharmacological intervention (Martin Wilson, personal communication). Alternatively, the localisation may result from antigen saturation in the target tissue of the IMHV such that all the R14 immunoglobulin is "mopped up" by an excess of antigens close to the injection site preventing any further diffusion. We could determine this experimentally by the injection of similar quantities of antibody which we knew did not bind to chick brain, if the localisation of R14 results from antigen saturation then a non-anti-brain antibody should diffuse further through the brain.

The time-course of the effect of R14 (amnesia at 24hrs but not at 1hr or 3hrs following training) suggests that the antiserum is interfering with a long-term memory process while leaving earlier mechanisms intact. These results are at odds with conventional models of memory formation in the chick in which long-term memory is said to be already established by 3hrs post-training (e.g. Patterson et al., 1986;1988). However, results which have emerged in recent years, both from our laboratory and from other researchers, may be relevant to the findings of this study.

In our own laboratory a modification of a conditioned taste-aversion task has been developed in which chicks are presented with a dry, coloured bead and, 30min later, injected intraperitoneally with the emetic lithium chloride. Chicks tested 3hrs following "training" avoid the original bead while pecking at a different coloured bead (Barber et al., 1989). As well as posing questions about the conventional premise of Hebbian models of near-simultaneous pre-post synaptic firing and stimulus presentation, this experiment suggests that, immediately following training, a certain amount of redundant or superfluous information is coded, i.e. pecking at the neutral training bead has no biological consequences for the animal until 30min after the event.

Further evidence for the above hypothesis comes from experiments in which a comparison was made between chicks trained on a bead coated in methyl anthranilate with one coated with another aversant, quinine. Groups of chicks were tested at several time-points following training and the results showed that while quinine-trained and MeA-trained animals showed similar levels of retention at 45min, thereafter retention in the quinine-trained animals declined progressively to below that for MeA chicks by 3.5hrs and 12hrs and were statistically similar to water-trained controls by 24hrs (Bourne et al., 1991). These findings are consistent with a model in which pecking at the quinine bead does not provide a salient enough stimulus for the memory trace to remain consolidated in a "very long-term" store. Instead it appears that, in the case of the quinine-trained birds, the memory trace decays progressively after an early consolidation period.

Consistent with the above behavioural data, there is increasing evidence of a "second wave" of memory-related biochemical processes, similar to those occurring at early time points, but taking place some 6 to 10hrs following training. In our own laboratory we have found that the fucose analogue 2-deoxygalactose (2-D-Gal) is amnestic when administered within a time window 2hrs before to 2hrs following training (Barber et al., 1989; see chapter 2). Outside this susceptibility window, the amnestic effect of 2-D-Gal rapidly decays and then rises again over subsequent hours until 6hrs post-training when, again, intracranial injections of the agent result in amnesia (Zamani and Rose, unpublished data). Using a passive-avoidance paradigm in the rat Regan and colleagues have demonstrated that intraventricular infusion of antibodies to the neural cell adhesion molecule (N-CAM) result in amnesia only when administered within a time window of 8 - 10hrs following training and not at any time-point other than this (Doyle et al., 1990).

Taken together, the experiments described above support a model in which training sets in motion a series of biological mechanisms, including the synthesis of synaptic membrane

proteins and glycoproteins. As well as coding biologically relevant information, these processes are involved in the immediate coding of a certain amount of redundant information and it is only some time later (approximately 6 - 10hrs) that a selective consolidation into "permanent" memory occurs. This second wave of activity may reflect a selection of synapses and a "stamping in" of relevant memory traces. It appears to include the synthesis of a class of fucosylated glycoproteins which is inhibited by 2-D-Gal in a fashion similar to that occurring near the time of training. Further support for this idea comes from Bourne et al.'s (1991) quinine experiments; incorporation of radioactive fucose into glycoproteins was elevated in MeA-trained but not quinine-trained animals. Also involved in the second wave are isotypes of N-CAM whose effect is inhibited by intraventricular infusion of anti-N-CAM antibodies 8-10hrs following training and, importantly, not inhibited by similar intervention at the time of training, at least in Regan's rat passive avoidance paradigm. This suggests that the second wave of consolidation includes the synthesis of a unique subset of glycoproteins only at the later time point. However, there is no reason to suppose that the first and second wave-related glycoproteins are all mutually exclusive species.

It is possible that the results reported in this chapter relate to the model reported above. The localisation of the antibody was restricted to the IMHV 1hr following intracranial injection. It is may be that by the time of the second-wave of glycoprotein synthesis, antibody has diffused throughout the brain to the point where it binds functional antigens in loci involved in the "stamping in" consolidation phase. The lesion studies described in the general introduction indicate that these loci include the LPO, since post-training but not pre-training lesions of this area result in amnesia (Patterson et al, 1990). Additionally, we cannot rule out the possibility of antibodies resulting in amnesia through binding to target tissue in some forebrain roof area as yet unidentified as playing a role in passive avoidance.

It should be noted, however, that such a mode of action cannot account for the finding that administration of antibody at 30min or less pre-training does not produce amnesia. In fact, the most straightforward interpretation of these data is that injections of R14 IgG result in the slow diffusion of antibodies which take at least 60min to bind to their respective epitopes and inhibit a sufficient number of functional antigens in the IMHV at, or very soon after, the time of training. The inhibited antigens are involved in a memory-related process which is set in motion very soon after training but is expressed behaviourally only after at least 3hrs. However, this explanation conflicts with data from lesion studies which indicate that the IMHV is necessary for early acquisition but not for retention after 3hrs (Patterson et al., 1990). It is, of course, possible that the antibodies may be causing amnesia by binding target antigens in a non-IMHV forebrain roof area, not yet identified as playing a role in passive avoidance learning.

There is evidence from other sources that injected antibodies remain active for some time. Antiserum injected into grasshopper eggs remained active for 24hrs and over the following days labelling persisted although with decreasing intensity (Petrey et al., 1989). Tissue sections from neonatal rats taken 48hrs after they had been injected intraperitoneally with anti-nerve growth factor displayed heavy antibody staining (Hulsebosch and Fabian, 1989).

The amount of amnesia resulting from intracranial injections of the antiserum (approximately 50% of chicks) is not as high as that observed for other amnestic agents such as the protein synthesis inhibitor, anisomycin or the fucose analogue, 2-deoxygalactose. However, no dose-response curve has yet been established for R14 so it is possible that injections at different doses and at time-points longer than 60min before training may be more effective in producing amnesia. It should be noted that the amount of amnesia is similar to that reported for other anti-brain antibodies (see introduction to

this chapter). Furthermore, it is unlikely that all six of the antigens recognised by R14 (see chapter 3) are involved in memory processes. Consequently, non-amnestic antibodies contained within the antiserum would effectively dilute the effect of those which interfere with memory formation. This problem could possibly be addressed by the isolation of antibodies to each individual antigen in order to establish which are specifically involved in synaptic plasticity (see general discussion).

The question of the mode of effect of R14 is not clear, but there are several possibilities which are worth consideration (see figure 4vi). One possibility is that the antibody enters the cell and disrupts cellular mechanisms by binding to intracellular antigens. It has, however, been reported that immunoglobulins are incapable of passing the plasma membrane (Karpiak and Rapport, 1975) and even though, theoretically, antibodies may enter cells by pinocytosis, once inside they would be rapidly degraded by lysosomal enzymes. A more likely explanation is that the amnestic effect is the result of antibodies binding to cell-surface macromolecules. It is tempting to speculate that the antibodies "cap" synaptic proteins, thereby disrupting their function by masking functional epitopes or by inhibiting their movement through the membrane (see general discussion). The size of the synaptic cleft (~20nm) would allow the entry and movement of immunoglobulins whose size is estimated at 10-15nm (with a large amount of flexibility of shape about the hinge region).

It is also possible that the disruption of memory formation is due to binding to, for example, neurotransmitter receptors or to non-synaptic ion channels (see figure 4vi). It is worth noting that the antigens which R14 recognises are enriched in membranous, and particularly synaptic, fractions (see Section 3viii). We do not know, of course, that the antigens recognised by R14 are located on the cell surface. This could be addressed by immunohistochemistry (although how much relation treated tissue bears to that *in situ* is

Hypothesised actions of amnestic antibodies

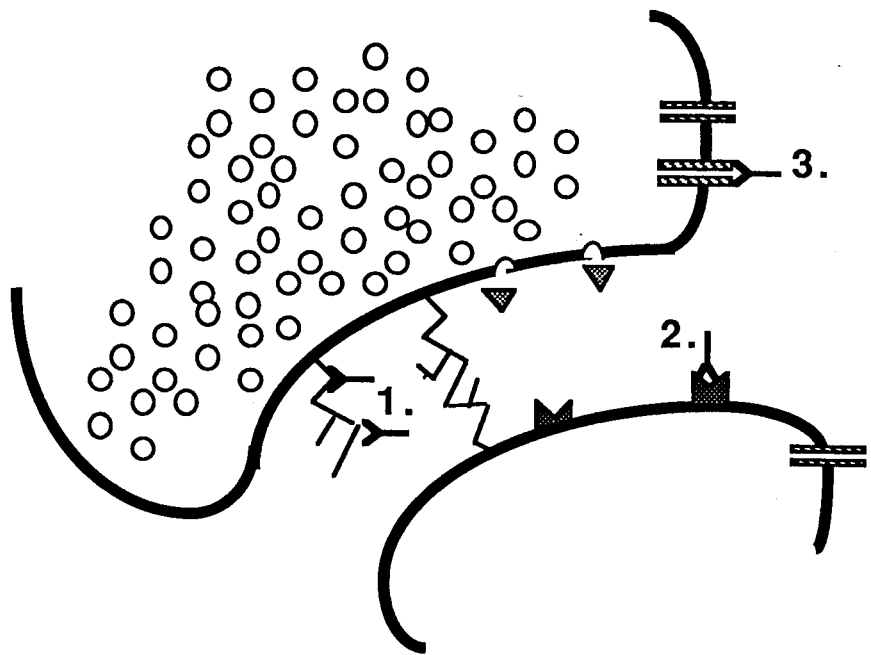


Figure 4vi) Schematic diagram of hypothetical actions of amnestic antibodies. Possible mechanisms of interfering with synaptic plasticity include
1. Binding to synaptic glycoproteins and preventing movement through the membrane or insertion of new carbohydrate. 2. Binding to neurotransmitter receptor. 3. Disruption of ion channel function (see text).

unclear), or by radiolabelling the cell surface (e.g. by iodination), immunoprecipitating with R14 and checking for the accumulation of radiolabel within the antibody-antigen complexes.

CHAPTER 5: CHANGES IN CYTOSKELETAL PROTEINS FOLLOWING PASSIVE AVOIDANCE LEARNING.

INTRODUCTION.

Having demonstrated that intracranial injections of an anti-PSD protein are capable of disrupting memory formation, we wished to examine the possibility of antibodies being used to probe for changes in brain tissue following passive avoidance training. Although ideally one would like to use the same antibody to disrupt neural function and to probe for plastic changes following training, the heterogeneity of antigens which R14 recognises precluded this possibility. However, the availability of antibodies to the cytoskeletal proteins tubulin and microtubule-associated protein 2 (MAP2) provided an opportunity to look for changes in levels of these proteins following training.

The neuronal cytoskeleton is a three-dimensional network which determines cell shape, including synaptic and dendritic morphology. The cytoskeleton is potentially involved in many biological events which may underly synaptic plasticity. Being dynamic, it is likely to be involved in determining neuronal morphology. The cytoskeleton is also involved indirectly in many cellular processes including neurotransmission through its scaffolding and transport functions which support transducing proteins such as enzymes and receptors.

Tubulin is the major constituent of microtubules which, along with microfilaments and intermediate filaments, constitute the cytoskeletal "scaffolding" of the cell. Any cellular reconstruction or increased demand for cellular traffic which plays a part in memory formation might reasonably be expected to involve changes in both amino acid

incorporation into and expression of tubulin. Indeed, an early study from our laboratory found that both the incorporation of leucine into tubulin (or "colchicine-binding protein") and absolute levels of the protein were increased dramatically following chick passive avoidance learning (Mileusnic et al., 1980, see below).

Microtubules are hollow cylinders (inner diameter approximately 15nm, outer diameter approximately 25nm) composed of tubulin dimers (molecular weight 110kDa) each comprising an α and a β monomer, which on PAGE migrate as two distinct polypeptide species of M.W. approximately 56kDa and 54kDa, respectively. Millimolar concentrations of Mg^{2+} are required for the polymerisation of tubulin into microtubules whereas micromolar Ca^{2+} inhibits polymerisation. In vitro injection of Ca^{2+} at micromolar concentrations induces microtubule disassembly, possibly through the synergistic action of microtubule-associated protein 2 and Ca^{2+} /calmodulin (Keith et al., 1983; Margolis, 1983).

Microtubule-associated proteins (MAPs) are a group of proteins which co-polymerise with purified brain microtubule preparations. They are involved in the regulation both of neuronal shape and of the balance of rigidity and plasticity in neuronal processes (Matus, 1987). MAP2 exists as a doublet polypeptide with a molecular weight of approximately 280 kDa, and a smaller subunit, MAP2c, of 70kDa. It stimulates the assembly of microtubules in vitro (Murphy & Borisy, 1975) and is thought to mediate the interaction of microtubules with other cell components (Bloom & Vallee, 1983; Aamodt & Williams, 1984). This interaction appears to be controlled by phosphorylation; MAP2 harbours a major fraction of the cAMP-dependent protein kinase (Theurkauf and Vallee, 1983) to the extent that this enzyme has been described as a microtubule-associated-protein-associated-protein or "MAPAP" (Schliwa, 1986). The molecule is also phosphorylated by the Ca^{2+} /calmodulin dependent kinase (Goldenring et al., 1985), casein kinases (Risnik et

al., 1988) and protein kinase C (Hoshi et al., 1988).

Trypsin and chymotrypsin digestion of MAP2 cleaves it into a small fraction of 32-39kDa and a large one of 240kDa. The smaller peptide retains the microtubule polymerising capacity of the intact protein (Vallee, 1980) while the larger fragment forms the microtubule projection domain that appears to be involved in mediating the interaction with other cell components (Vallee, 1984).

A number of studies have reported changes in cytoskeletal proteins following various manipulations of experience which may result in plastic changes. Tubulin has been shown to change in both measures of precursor incorporation into tubulin and absolute levels of the protein following light exposure of dark-reared rats (Perry and Cronly-Dillon, 1978). Previous work in our laboratory has identified an increased incorporation of [³H]lysine into a polymerisable fraction of the rat visual, but not motor, cortex following eye-openig (Rose et al., 1976).

Both in vivo and in vitro colchicine binding assays, a measure of the amount of tubulin, revealed a transient increase in the total amount of colchicine-binding protein in a particulate fraction in light-exposed compared to dark reared animals (Stewart and Rose, 1978). Additionally, there is a change in both total amount of, and precursor incorporation into tubulin of the forebrain roof following imprinting in the chick (Longstaff and Rose, unpublished experiments).

The experiment reported here made use of an antibody to α -tubulin. The RNA message for, and the total levels of the α subunit has been shown to increase following transient application of the adenylate cyclase activator, forskolin, to the dorsal root ganglion of the frog (Carlsen et al., 1990). The molecule is also developmentally-regulated (Havercroft

and Cleveland, 1984; Tetzleff and Bisby, 1990) and has been implicated in nerve regeneration (Hoffman and Lasek, 1980; Tetzleff and Bisby, 1990).

With respect to the passive avoidance paradigm, Mileusnic et al. (1980) identified an increase in both precursor incorporation into, and total quantity of tubulin following training. Mileusnic's study demonstrated increased levels of tubulin in the anterior portion of the chick forebrain roof, but not the posterior portion of the roof nor in the base, following passive avoidance training. The increase in tubulin levels was significant at 30min post-training and persisted for at least 24hr but had returned to control levels by 48hr post-training. The elevation in tubulin content was found in both a particulate fraction and a postmitochondrial supernatant.

Immunohistochemical studies have revealed that brain MAP2 is expressed almost exclusively in dendrites (Bernhart & Matus, 1984; Burgoyne & Cumming, 1984), where it is also synthesised (Garner et al., 1988). In cerebellar basket cells, MAP2 is present in the axon but does not extend beyond the initial portion and thus its neuronal distribution cannot be due to selective transport into dendrites (Bernhart & Matus, 1984). Cytoplasmic partitioning of MAP2 has also been described in cultured neurons, which rules out the possibility of extrinsic factors influencing its intracellular distribution (Matus et al., 1986). Dispersed hippocampal pyramidal cell cultures contain MAP2 within their dendrites but not their axons (Matus et al., 1986), although in these cultures MAP2 is initially also present in axons. In cultures of cerebellar granule cells MAP2 is expressed throughout the entire length of neurites as they grow out of the cell body and only later becomes restricted to dendrites (Alaimo-Beuret & Matus, 1985).

Until recently it was believed that MAP2 functioned exclusively as a regulator of microtubule assembly, however a study by Bernhart et al. (1986) found that, at least in

Purkinje cells, the molecule goes through developmental stages where it does not have the filamentous appearance that would be expected if it were microtubule-bound. Simultaneous, double-label immunofluorescence of both MAP2 and tubulin of hippocampal cells in dispersed culture revealed a differential distribution of the two proteins. MAP2 staining was observed throughout the cytoplasm while tubulin maintained its filamentous appearance (Matus et al, 1986). If microtubules of cultured cells were de-polymerised by nocodazole treatment, the de-polymerised tubulin exhibited the same distribution as MAP2. At the same time multiple side branches emerged along the dendrites (Matus et al.,1986). These results suggest that dendritic MAP2 exists at levels in excess of its tubulin binding sites and that under normal conditions it acts to ensure total polymerisation of microtubules and to suppress adventitious side-branching.

Perhaps the most important characteristic of MAP2 is the differential expression of the high and low molecular weight isoforms during development. The 70kDa MAP2 abounds during embryogenesis and disappears by adult age, the 280kDa form undergoes a reciprocal expression (Nunez, 1988).

The aims of the following experiments are twofold. Firstly, we wished to determine if the loci of changes in levels of tubulin at various time points post-training could be more precisely determined using the relatively sensitive methods of antibody technology. Secondly, we wished to see if the levels of MAP2, a protein which appears to play a crucial role in developmental plasticity, were changed following passive avoidance training in the young chick.

METHODS.

5i) Training.

Chicks were trained in pairs as described in section 4ii). Both animals in each pen were presented with either a water-coated bead (W birds) or a bead coated with methylantranilate (M birds).

Antibodies.

Monoclonal antibody YLT/2 which specifically recognises the tubulin α monomer was a kind gift from Dr. John Kilmartin of the Medical Research Council Laboratory of Molecular Biology in Cambridge. The specificity of the antibody is indicated in the Western Blot in figure 5i).

The anti-MAP2 antibodies were a gift from Andrew Matus of the Friedrich Miescher-Institut, Switzerland. Both were monoclonal antibodies; one ('C') binds to an epitope of dendritic and axonal origin in the developing nervous system, which becomes compartmentalised to the dendrite by birth, and recognises the 280 kDa polypeptide and a smaller 70 kDa subunit. The other ('AP14') recognises only the high molecular weight, 230kDa subunit and binds only to dendrites.

Typical Western Blots of the anti-tubulin and titrations of all the antibodies are presented in Figures 5i), 5ii) A and B. (see sections 3iv) and 3vii) for details of procedures)

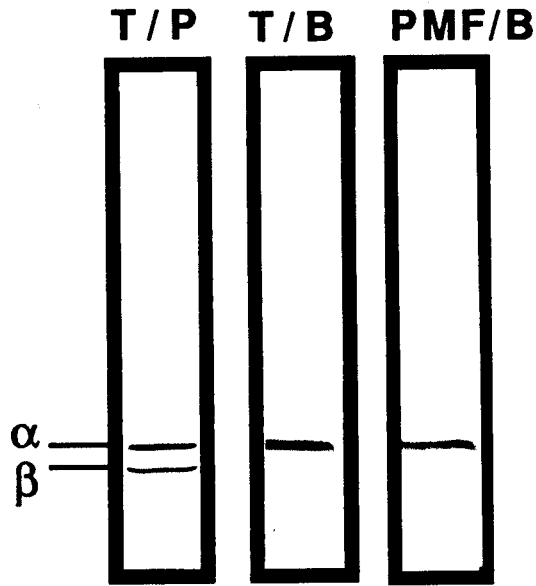


Figure 5i) SDS-PAGE and Western Blot using anti-tubulin. T/P = tubulin preparation stained with coomassie blue (α and β monomers are indicated), T/B and PMF/B = tubulin preparation and postmitochondrial fraction respectively, blotted and probed with anti- α -tubulin.

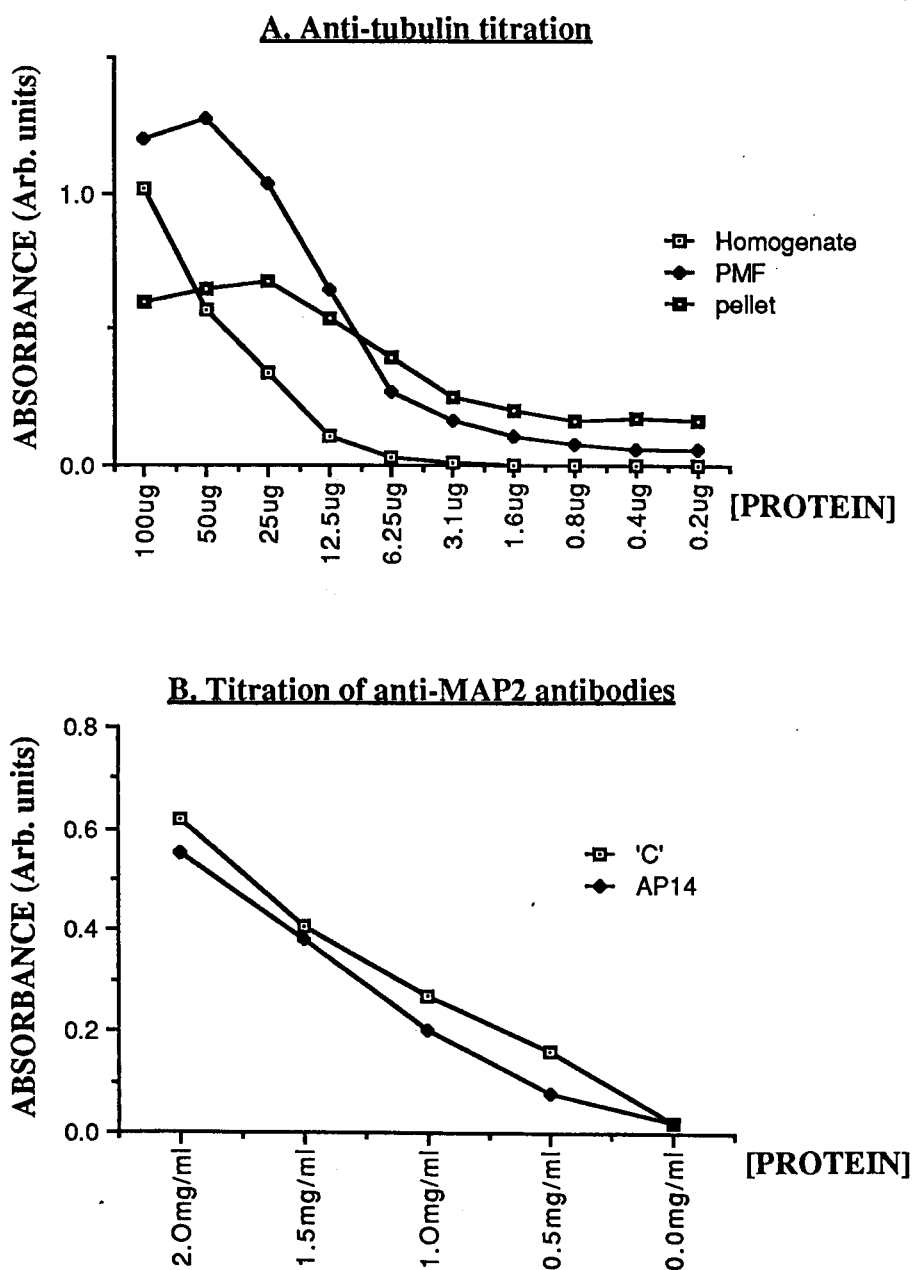


Figure 5ii) A. Titration of anti-tubulin using various protein concentrations of parent homogenate (hom), particulate fraction (pellet) and postmitochondrial (PMF) fraction. B.Titration of anti-MAP2 antibodies 'C' and AP14 using crude forebrain homogenate as antigen.

5ii) Testing and Tissue Preparation: Tubulin Assays.

1hr, 6hr or 24hr following training, W birds which pecked at a dry chrome bead and M birds which avoided the bead on testing (N = 16 in each condition at each time-point) were killed and the brains immediately removed. Six brain loci, the left and right IMHV, PA and LPO were dissected out on ice using the resin brain mould exactly as described in section 4vi).

To provide enough material for the preparation of the fraction to be used for the tubulin assays, pairs of brain loci were pooled each from two birds which had undergone the same training procedure, thus yielding eight samples for each training condition from all three post-training time points. The dissected loci were homogenised by sonication in 1ml of 20mM Na-phosphate (pH 6.8), 0.32 M sucrose, 5mM MgCl₂, 0.1mM GTP and centrifuged at 16,000gmax for 30min. Aliquots of the postmitochondrial supernatant and the particulate pellet were taken for protein determination. In this study only postmitochondrial fractions were used for immunoassays because it proved difficult to obtain a satisfactory titration using the particulate fraction as antigen (see figure 5ii). Additionally, the postmitochondrial fraction is largely cytosolic and subcellular localisation of the changes found in Mileusnic's study revealed that the particulate fraction changes were exclusive to the synaptosol with no changes being found in the membrane fraction. Portions of postmitochondrial fractions were diluted in PBS (pH 6.8), 5mM MgCl₂, 0.1mM GTP to appropriate concentrations (10 - 50µg/ml protein) for immunoassay.

5iii) Testing and Tissue Preparation: MAP2 Assays.

1hr or 24hrs post-training, W birds which pecked the bead and M birds which avoided the bead on presentation were killed and the six forebrain loci removed immediately on ice. Tissue from each forebrain region was quickly homogenised by sonication in 500 μ l of ice-cold lysis buffer (5mM Tris-HCl, pH 8.3) containing 1mM protease inhibitor, phenylmethylsulphonylfluoride (PMSF). This provided four sets of samples: regions from W birds taken 1 hr and 24hrs post-training (N = 19 and 24 respectively, and those taken from M birds 1hr and 24 hrs post-training (N = 18 and 23 respectively). The protein content of each of the six brain regions from each group was determined and each was diluted to appropriate concentrations (1 ± 0.25 mg/ml protein) for immunoassay.

5iv) Immunoassays.

Antigen levels in homogenates of the forebrain regions were determined using a standard enzyme-linked immunosorbant assay (ELISA) performed in 96-well microtest plates (see Section 3v) for details). Anti-tubulin was used at a dilution of 1:1000 in PBS/tween. Both AP14 and C were used at dilutions of 1:50 (found to be the most suitable dilution in pilot experiments). The second antibody was horseradish peroxidase conjugated to affinity purified goat F(ab)₂ anti-rat IgG (gamma and light chain specific) for the anti-tubulin or anti-mouse IgG for the anti-MAP2s. The substrate used was *o*-phenylene diamine, a chromagen which absorbs at 492nm. All samples were assayed in triplicate and absorbance readings were divided by protein content as determined by Bradford's method (Bradford, 1976).

RESULTS.

Regional Changes in Anti-tubulin titre.

The results of the tubulin assays are presented in figure 5iiii) and in Table 5i). There was an increase in the titre of anti-tubulin in the left IMHV of trained birds 1hr (+55%; $p < 0.01$) following training which persisted and was significant compared to controls at 6hrs (+58%; $p < 0.05$) and 24hrs (+49%; $p < 0.01$) post-training. Tubulin levels in the left LPO (+49%; $p < 0.05$) are significantly elevated at 1hr but not at 6hrs or 24hrs. Conversely, in the right LPO, tubulin levels were not significantly different in M birds compared to W birds at 1hr post-training but were elevated at 6hrs (+82%; $p < 0.05$) and 24hrs (+ 46%; $p < 0.05$). No other changes in any region examined differed significantly between W and M birds.

Brain region		Time post-training		
		1hr	6hr	24hr
left IMHV	W	0.199 (0.015)	0.293 (0.084)	0.224 (0.027)
	M	0.308 (0.030)**	0.461 (0.065)*	0.334 (0.019)**
right IMHV	W	0.281 (0.033)	0.345 (0.059)	0.255 (0.024)
	M	0.262 (0.033)	0.332 (0.031)	0.235 (0.014)
left LPO	W	0.206 (0.023)	0.284 (0.027)	0.195 (0.038)
	M	0.308 (0.035)*	0.418 (0.072)	0.210 (0.029)
right LPO	W	0.230 (0.025)	0.217 (0.020)	0.217 (0.032)
	M	0.256 (0.045)	0.397 (0.064)*	0.318 (0.027)*
left PA	W	0.233 (0.027)	0.321 (0.042)	0.233 (0.022)
	M	0.265 (0.013)	0.307 (0.022)	0.223 (0.037)
right PA	W	0.233 (0.020)	0.247 (0.028)	0.236 (0.041)
	M	0.286 (0.034)	0.367 (0.065)	0.262 (0.024)

Table 5i) Mean levels of anti-tubulin immunoreactivity per total protein concentration (arbitrary units) in brain regions dissected out 1hr, 6hrs or 24hrs following passive avoidance training. Standard errors are presented in parentheses, N = 8 samples (16 birds) in each group (*, $p < 0.05$; **, $p < 0.01$ as determined by one-tailed student t-tests).

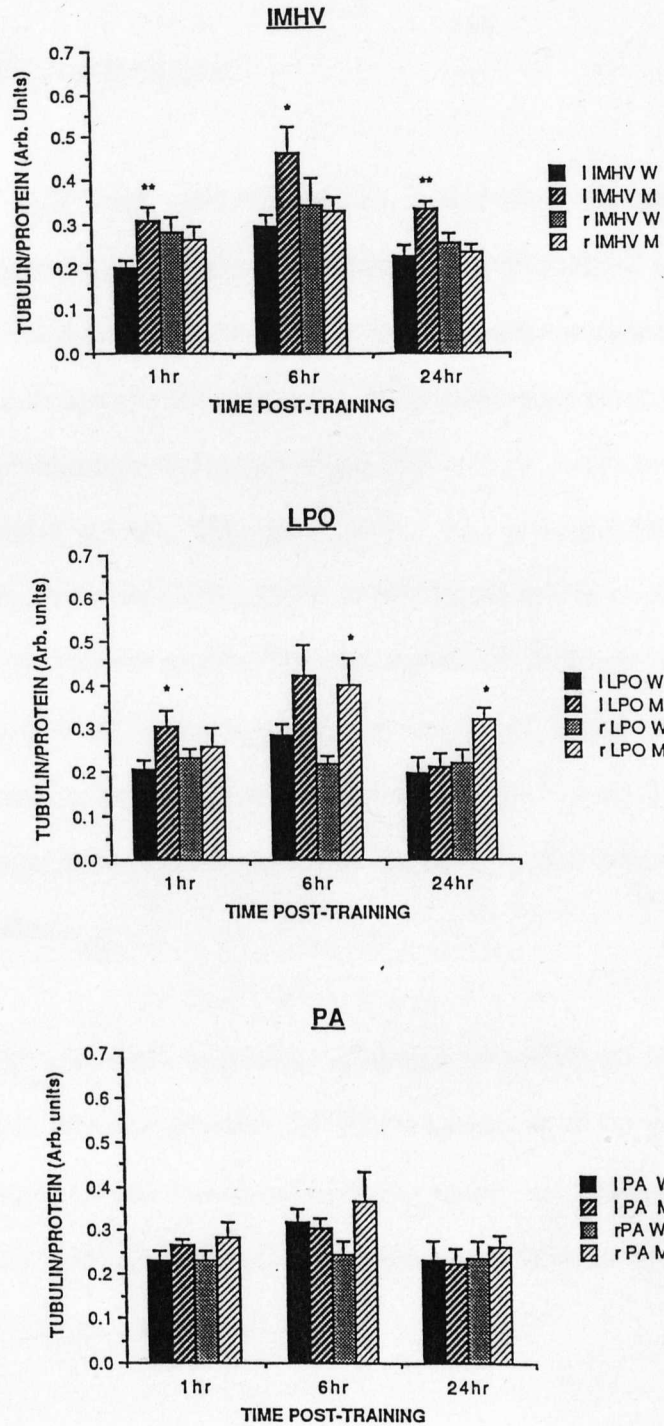


Figure 5iii) Mean levels of anti-tubulin immunoreactivity measured as absorbance/protein concentration (arbitrary units) in three forebrain loci taken 1hr, 6hrs or 24 hrs post-training from W and M chicks. Error bars are standard errors (**, $p < 0.01$; *, $p < 0.05$ as determined by one-tailed student t-tests).

Regional Changes in Anti-MAP2 Titre

The changes in anti-MAP2 titre were less clear cut than those for the anti-tubulin as there was a considerable amount of variation in the data. No significant differences were found between W and M birds in any of the individual brain regions examined (see table 5ii). If, however we measured (arbitrary absorbance units)/(protein concentration), calculated as left hemisphere/right hemisphere for each brain region from every animal, inter-animal variability was reduced greatly. This gave twelve data points: left/right hemisphere readings for the three brain loci LPO, IMHV and PA from both W and M birds, both 1hr and 24hr post-training (data not shown). The only significant difference between M and W birds was in binding of 'C' to homogenates of the IMHV 24hrs following training. Although this appeared to be a rather contrived way of manipulating the data, it was felt that the result merited further investigation into the binding of monoclonal antibody, 'C', in the IMHV (see below).

Student's t-tests were also performed to examine the possibility that the birds undergo significant developmental changes over the 24hr time course of the experiment, or that pecking at the methylanthranilate-coated bead may evoke such changes. No significant changes were found in antibody binding to any brain region between the two time points.

		AP14		'C'	
		Time post-training			
Brain region		1hr	24h	1hr	24hr
left IMHV	W	0.857 (0.167)	1.261 (0.191)	0.880 (0.175)	1.087 (0.143)
	M	0.830 (0.175)	1.015 (0.121)	0.905 (0.194)	0.892 (0.086)
right IMHV	W	0.751 (0.139)	1.174 (0.141)	0.754 (0.152)	1.023 (0.110)
	M	0.924 (0.179)	1.232 (0.185)	0.899 (0.158)	1.042 (0.136)
left LPO	W	0.795 (0.106)	1.207 (0.129)	0.819 (0.113)	0.940 (0.085)
	M	0.884 (0.131)	1.155 (0.104)	0.866 (0.118)	1.155 (0.936)
right LPO	W	0.875 (0.151)	1.376 (0.165)	0.868 (0.147)	1.074 (0.125)
	M	0.838 (0.120)	1.214 (0.0129)	0.862 (0.104)	0.936 (0.077)
left PA	W	0.898 (0.171)	1.411 (0.247)	0.894 (0.196)	1.102 (0.150)
	M	1.048 (0.233)	1.303 (0.189)	1.004 (0.228)	1.303 (0.120)
right PA	W	1.000 (0.159)	1.356 (0.194)	1.044 (0.188)	1.048 (0.099)
	M	0.920 (0.158)	2.154 (0.582)	0.938 (0.174)	2.154 (0.292)

Table 5ii) Mean levels of immunoreactivity of anti-MAP2 monoclonals 'C' and AP14 in brain regions of the chick brain taken 1hr or 24 hrs following passive avoidance training on an M or a W bead. Standard errors are presented in parentheses (N = 18 - 24 in each group).

5v) Time course of changes in MAP2.

The aim of this experiment was to establish if changes in the binding levels of 'C' could be detected at intermediate time points following passive avoidance learning and to determine if the putative 24hr change in 'C' titre was contributed to by an elevation in the right IMHV or by a decrease in the left IMHV.

Procedure.

Chicks were trained exactly as described above, dissections were made only of left and right IMHV of M birds which avoided the bead and W birds which pecked at the bead, at 1, 6, 12 or 24hrs following training (N = 11 - 13 in each group). The procedure for ELISA was exactly as described above.

Results.

The mean immunoreactivity of the left and right IMHVs from the timecourse experiment are presented in Table 5iii) and graphically in Figure 5iv) in which all immunoreactivities per region are divided by the W value for that region, so that W values are equal to unity.

Although there were no statistically significant differences there is a clear trend for the levels of 'C' immunoreactivity to increase above controls in both hemispheres at earlier time-points and for the titre to decrease below W levels at 24hrs post-training in the left IMHV rather than to become elevated in the right IMHV. This result suggests that the putative significant difference in the left hemisphere/right hemisphere index 24hrs

following training in the passive-avoidance paradigm results in a decrease in either absolute level or accessibility of the MAP2 epitope recognised by the monoclonal 'C'.

	Region			
	left IMHV		right IMHV	
	W	M	W	M
Post-training time				
1hr	1.072 (0.124)	1.224 (0.170)	1.008 (0.131)	1.243 (0.138)
6hr	1.108 (0.140)	1.341 (0.182)	1.162 (0.170)	1.302 (0.192)
12hrs	1.222 (0.093)	1.065 (0.110)	1.182 (0.139)	1.116 (0.126)
24hrs	1.107 (0.113)	0.966 (0.070)	1.084 (0.107)	1.129 (0.100)

Table 5iii) Mean levels of immunoreactivity of anti-MAP2 monoclonal antibody, 'C' in IMHVs taken 1hr, 6hrs 12hrs or 24 hrs following passive avoidance training on an M or a W bead. Standard errors are presented in parentheses (N = 11 - 13 in each group).

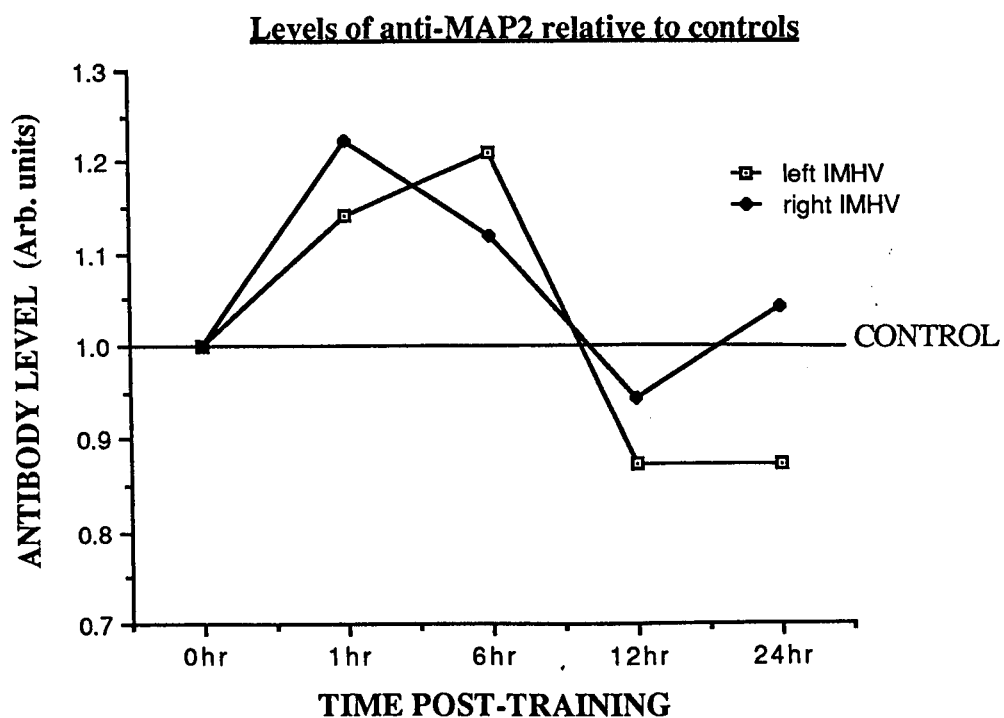


Figure 5iv) Mean values for the timecourse experiment showing monoclonal antibody 'C' binding to left and right IMHVs taken 1hr, 6hrs 12hrs and 24hrs post-training relative to a control value adjusted to 1.0.

DISCUSSION

The results presented above confirm earlier findings that passive avoidance training in the chick results in elevated levels of brain tubulin within a postmitochondrial fraction. In this study, training-related increases in tubulin were further localised and to the left IMHV and left LPO 1hr following training, and the left IMHV and the right LPO at 6hrs and 24hrs post-training.

These results are compatible with those of Patterson and Gilbert obtained from their lesioning studies (Patterson et al., 1990; Gilbert et al., 1991). They found that the left, but not the right IMHV is necessary for acquisition of memory, but that by 1hr post-training the IMHV is not necessary for further retention. These studies further demonstrated that the LPO is necessary for retention, but not acquisition, and that the LPO becomes important in this retention some time between 1hr and 24hrs post-training. The elevated levels of tubulin observed in this study may reflect an increase in the synthesis of tubulin in regions which are undergoing cellular reconstruction and/or an increased demand for intracellular traffic along dendrites and axons. Soon after training these areas seem to be the left IMHV and the left LPO but by 6hrs post-training, cells within the the right LPO have an increased demand for tubulin.

Although the lesioning studies have indicated that both left and right LPO may be involved in retention, this is based on the finding that unilateral lesions of either LPO are without effect on retention. There is some evidence that in the absence of one brain loci usually responsible for some particular function, other brain loci can take over that function. For example, if the right IMHV was lesioned before training, chicks show recall 3hrs post-training, if lesions are then made to the left IMHV and to both the left and right LPOs the chicks are not amnesic although both IMHVs and both LPOs are ablated

(Gilbert et al., 1991). This suggests that some structure other than the IMHV or LPO takes over the role of retention if lesions are performed in this manner. In the case of unilateral lesions to the right LPO it is possible that the left LPO takes over its responsibility for retention.

The persistence of elevated levels of α -tubulin in the left IMHV and the right LPO beyond times where the regions appear to be necessary for learning on the basis of lesioning may reflect a "carry over" of synthesis of the molecule following initial stimulation of the loci. There is indirect evidence for similar phenomena in different preparations. Transient application (for 1hr) of forskolin to neurons of the dorsal root ganglion of the frog resulted in a twofold increase in axonal transport 12hrs following the withdrawal of forskolin. This was accompanied by an increase in the amount of mRNA coding for the α subunit and in levels of α -tubulin determined by 2-D PAGE (Carlsen et al., 1990). It should also be noted that many of the biological changes found in the IMHV appear to persist until at least 24hrs post-training, for example an increase in dendritic spine density (Patel and Stewart, 1988; see below)

The changes found in this study are dramatic (ranging from 46% to 82% above that of controls) however, this may not reflect a percent-for-percent increase in total tubulin levels. There are four genes coding for each tubulin monomer in the chicken (Lopata et al., 1983), the reasons for this are unknown but there are two possibilities (Raff, 1984). Firstly, the tubulin gene-products may be similar and can co-polymerise into functional microtubules, each tubulin molecule possessing slightly different properties thus allowing slightly different modes of action. The second possibility is that the gene families arose from the need for differential regulation, that is to allow control of timing and location of tubulin synthesis. The two possibilities are not mutually exclusive and may operate to different extents in different cell systems (Cowan and Dudley, 1983). If the anti-tubulin

monoclonal antibody that we have used recognises an epitope which is exclusive to only one of the α -tubulin gene products then the elevation in the titre of the antibody may not reflect increases in gross levels of whole tubulin.

It should also be noted that in the earlier study of Mileusnic et al. (1980), the increases in levels of tubulin in the postmitochondrial fraction from the forebrain roof of M birds is of the order of 30%. Considering the very large size of the forebrain roof compared to the IMHV, if the left IMHV is exclusively responsible for the elevation of tubulin there must have been a considerable amount of dilution of tissue by areas which are not increasing tubulin synthesis as a result of training. This dilution factor may also account for the fact that Mileusnic's study found no changes in the forebrain base which contains the LPO, a locus which showed a dramatic elevation in this experiment.

With the respect to the MAP2 assays, the experiment described above indicated a possible asymmetric decrease in the binding of the monoclonal antibody C to crude homogenates of the IMHV in MeA-trained birds. If this result is a true reflection of a genuine biological change rather than merely a trend within the samples assayed then it may be interpreted in a number of ways. The monoclonal recognises a dendrite specific region of MAP2, and the result can be interpreted in a number of ways. One possibility is that the finding reflects an overall decrease in the level of MAP2, if this is true it is possible to speculate about the functional importance of such a decrease. Dendritic MAP2 exists at levels above that required to maintain polymerisation of microtubules (Bernhart & Matus, 1982; 1984) and when microtubules of developing cells in culture are artificially depolymerised, multiple adventitious side branches emerge from their dendrites (Matus et al., 1986).

These findings, along with the results of this experiment allow for the possibility that, during memory formation, MAP2 is degraded to a sub-threshold level, relaxing its

imposed structural rigidity on dendrites enough to allow for plastic changes in the postsynaptic structure. This may allow the memory-specific increase in spine density found in the left IMHV of MeA-trained birds (Patel & Stewart, 1988; Patel et al., 1988) when the sub-threshold level of MAP2 is coupled with changes in presynaptic activity, for example increased release of neurotransmitter. This possibility is, of course purely speculative, but there is at least a little evidence to support it. MAP2 is an excellent substrate for calpain I (Klein et al., 1981; Baudry et al., 1983) a calcium-activated protease which is believed to be involved in mediating synaptic plasticity.

In fact, a very similar model has been proposed as playing a part in long-term potentiation (e.g. Siman, Baudry and Lynch, 1987; Lynch and Baudry, 1987) and as a molecular substrate for long-term memory formation in a rather speculative hypothesis proposed by Friedrich (1990). In such hypotheses MAP2 and other cytoskeletal components are degraded by the action of the Ca^{2+} -dependent protease calpain which results in changes in dendritic morphology and therefore synaptic efficacy.

A less ambitious interpretation of the MAP2 data would be that, as the two monoclonals used in the experiment inhibit tubulin polymerisation (Matus et al., 1987) (suggesting that they bind to, and block, a functional site on MAP2) it follows that the asymmetric decrease observed in this experiment may reflect an increase of MAP-to-microtubule binding in vivo. This would be the result of some unknown mechanism mediating the restructuring of dendrites as a concomitant of memory formation. Alternatively, the decrease in the MAP2 epitope may reflect a conformational change in the molecule as a result of some change in post-translational modification such as phosphorylation. It has recently been demonstrated that activation of the NMDA receptor results in rapid dephosphorylation of MAP2 (Halpain and Greengard, 1990).

The results in this study may be worth considering in view of the fact that both molecules examined in these experiments are developmentally-regulated. There are marked changes in the expression of MAP2 during embryogenesis (Nunez, 1988; see introduction). With respect to α -tubulin, the expression of the mRNA for this molecule peaks at a time coincident with maximum synaptogenesis (Havercroft and Cleveland, 1984; Bhattacharya and Sarhar, 1991). Additionally, during nerve regeneration in response to axotomy, there is a re-expression of the developmentally-regulated isotypes of tubulin (Hoffman and Lasek, 1980; Tetzleff and Bisby, 1990). These findings lend support to the hypothesis that during memory formation there may be some kind of processes in play which are very similar to those observed during the development of the nervous system.

CHAPTER 6: CHANGES IN THE TITRE OF R14 AND 411B FOLLOWING LTP.

INTRODUCTION.

In the preceeding chapters it was established that intracranial injections of antibody are capable of producing amnesia for passive avoidance training, and that antibodies to neural antigens are useful tools in determining the loci and time-course of training-related biochemical changes in this paradigm. We went on to test the possibility of using antibodies to find loci-specific and time-dependent changes following another form of neuronal plasticity in a different animal, namely long-term potentiation (LTP) in the rat (see chapter 1 for a full discussion of LTP). There were several reasons for this shift in direction: partly the work was a continuation of an ongoing collaboration between our own laboratory and a group in Magdeburg, Germany (see below); the possibility of neural antigens which are conserved between the chick and the rat being involved in synaptic plasticity using different paradigms is of obvious interest to the neurobiologist, and finally we wished to see if changes in such antigens were detectable in an older animal - an approach not possible in the chick.

Previous work in our laboratory has resulted in the generation of a monoclonal antibody, 411B, which recognises an epitope of ~170kDa that is highly enriched in PSDs (Bullock et al., 1988). Collaborative studies between our own group and Bernd Loessner of the Institute of Pharmacology and Toxicology in the Magdeburg Medical Academy, Germany have revealed that, in the rat, the immunoreactivity of this monoclonal changes in response to haliperidol-induced dopaminergic supersensitivity (Loessner et al., 1988) and following LTP (Bullock et al., 1990) (see below).

During the characterisation of R14, it was found that the antiserum reacts monospecifically with a single band of ~230kDa on Western Blots of rat hippocampal tissue. The specificity of this recognition is interpreted as reflecting the phylogenic similarity of the rat compared with the host, rabbit. That is, antigenic determinants of similar proteins within the PSDs of chick and rat may have changed slightly during evolutionary diversification to the extent that they are antigenic to the rabbit when isolated from the chick but not so in the rat.

The monoclonal antibody, 411B, was raised against PSDs from 12-week old chicks. It recognises an antigen of molecular weight ~170kDa which is enriched ~13-fold in SPMs and ~24-fold in PSDs as compared to the parent homogenate from chick forebrain (Bullock et al., 1988) and, in the rat brain, is enriched in SPMs and PSDs by ~20-fold and ~60-fold respectively (Loessner et al., 1988). The usefulness of such antibodies has been considered by Beesley (1989) who states that they may recognise antigens which play unique and important synaptic functions, that they may be used as markers for synaptogenesis, synaptic stability and plasticity and that they may be useful for studies of biosynthesis of synaptic proteins. 411B is therefore considered to be a useful marker for plastic changes involving the PSD.

The PSD is an important component within the synaptic structure. It appears as a densely staining specialisation of the cytoskeleton lying adjacent to the postsynaptic membrane and has even been described as a "synaptic organelle" (Calverley and Jones, 1990). The postulated function of the PSD is in constricting the movement of membrane proteins, neurotransmitter receptors and ion channels in the part of the synaptic membrane to which it is attached (Matus, 1978; Cotman and Kelly, 1980). Proteins within the PSD include GABA receptors, glutamate receptors and various glycoproteins. The presence of these receptors along with channel proteins, kinases and their substrates (Wu et al, 1986)

suggest that the PSD is a dynamic structure capable of modulating synaptic transmission.

The structure of the PSD has been shown to change following a variety of experiential manipulations. Phillips (1985) reported an 11% increase in the length of cerebellar PSDs taken from rats which had been subject to a chronic alcohol diet followed by withdrawal. Rats which were rendered hypoxic for the first 17 days of life had significantly thinner PSDs compared to controls (Fischer et al., 1980). PSDs from young rats exposed to a tone for 24hrs had a significantly reduced cross-sectional area and mean thickness (Ross et al., 1985).

The number of PSDs in area CA3 of the hippocampus was increased in rats which were reared in a complex environment and had undergone maze learning. This difference was significant compared to controls which were reared in an impoverished environment or motor active controls (Altschuler, 1979).

With respect to LTP, changes in the numerical density and the morphology of the PSD have been reported following tetanisation of the perforant path (Desmond and Levy, 1986a; 1986b). These results are considered in detail in the discussion section of this chapter.

The protein recognised by 411B is believed to be glycosylated and there is evidence that the PSD is rich in high molecular weight glycoproteins (Gurd, 1985). There is further, indirect evidence, from electron microscope studies, that these glycoproteins project from the density and span the synaptic cleft where they are likely to be involved in post- to presynaptic interactions (Gray, 1966; Serchev, 1988). Clearly, changes in the amount or alterations in the functional carbohydrate moiety of glycoproteins have the potential to play an important role in the modulation of synaptic contact.

Rats which exhibited haloperidol-induced supersensitivity had increased immunoreactivity of 411B in the corpus striatum (+21%) and in the hippocampus (+45%) compared to saline-injected controls. By contrast, the titre of a monoclonal antibody, Q155, which recognises an integral synaptic vesicle protein was unchanged by haloperidol treatment (Loessner et al., 1988).

Of particular relevance to the experiments described in this thesis is the finding that the immunoreactivity of 411B is increased following LTP (Bullock et al., 1990). Tetanisation of the right perforant path resulted in a significant 34% increase in the immunoreactivity of tissue lysates taken from the target, ipsilateral dentate area compared to passive controls. No changes were found between controls and LTP animals in the other hippocampal subfields examined (areas CA1 and CA3) or in the titre of an anti-actin antibody. These results are considered in detail in the discussion section of this chapter.

Protein synthesis appears to play a ubiquitous role in long-term phases of memory formation (reviewed in Squire and Davies, 1984, see introduction). With respect to LTP, a late phase (i.e. longer than 3 - 5hrs following tetanisation) was shown to be inhibited by intraventricular infusion of anisomycin while tetanisation-specific electrophysiological responses at earlier time-points were unaffected (Krug et al., 1984). In addition, no effect of anisomycin was found on the slope of population excitatory postsynaptic potential (EPSP) recorded in non-potentiated synapses or on the amplitude of the population spike in these animals after the first few hours.

The aim of this experiment was to determine if LTP results in time- and region specific changes in levels of the synaptic macromolecules recognised by R14 and 411B. Further experiments were directed at determining the effect of intraventricular infusion of anisomycin on the observed increase in the titre of 411B 8hrs following LTP.

In the experiments described below, the tetanisation, dissection and anisomycin procedures were performed in Magdeburg by Professor Loessner's group. All other procedures were carried out by the author.

MATERIALS AND METHODS.

Antibodies.

R14: This antiserum was raised by chronic immunisation of rabbits with chick post-synaptic densities. The initial characterisation of this antiserum is described in chapter 3 of this thesis. Further characterisation revealed that it reacts strongly with a ~230kDa antigen in rat brain homogenates and a faint diffuse band of around 55kDa. Since the major reactive protein is synaptically enriched (see below) and is conserved, at least between chick and rat, it may stand as a good candidate for a protein involved in plastic changes in the nervous system. Typical Western Blots and titrations of R14 against chick forebrain homogenate and rat hippocampal homogenates are presented in Figures 6i) and 6ii) respectively.

411B: 411B is a monoclonal antibody raised against chick PSDs (Bullock et al, 1988) which recognises a 180kDa antigen again conserved between chick and rat (see introduction section).

An anti-actin antibody was also used in the LTP experiment as a non-selective control marker. A typical Western Blot of 411B and anti-actin is presented in Figure 6iii).

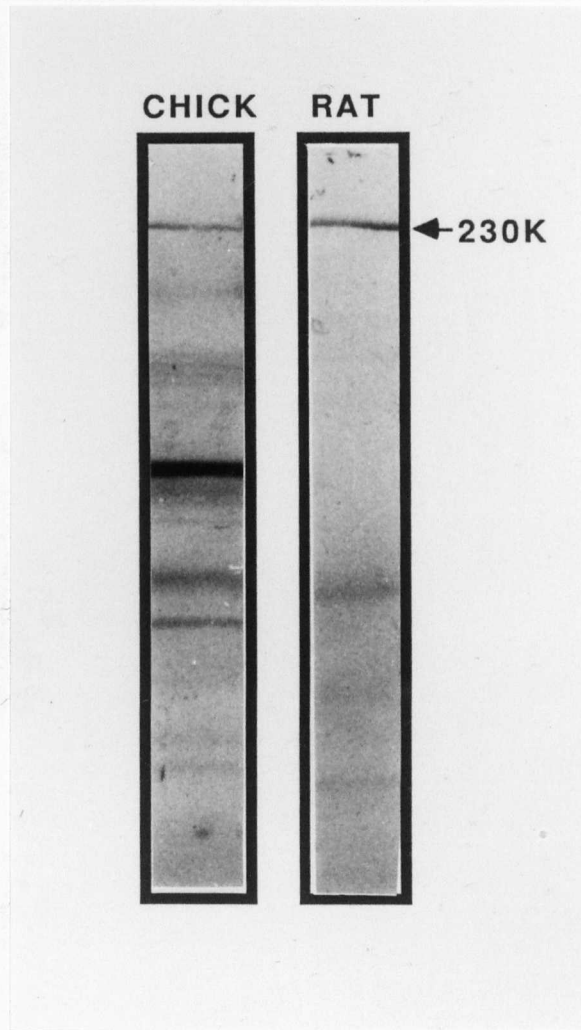


Figure 6i) Western Blot of R14 against chick forebrain homogenate and rat hippocampus homogenate.

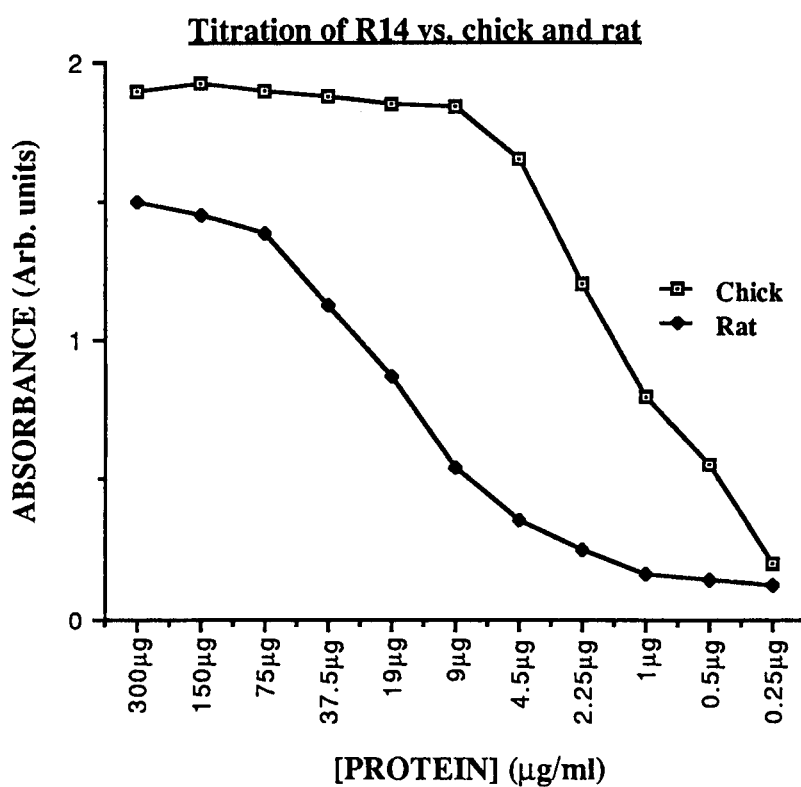


Figure 6ii) Titration of R14 showing binding levels to varying protein concentrations of chick forebrain and rat hippocampus homogenate.

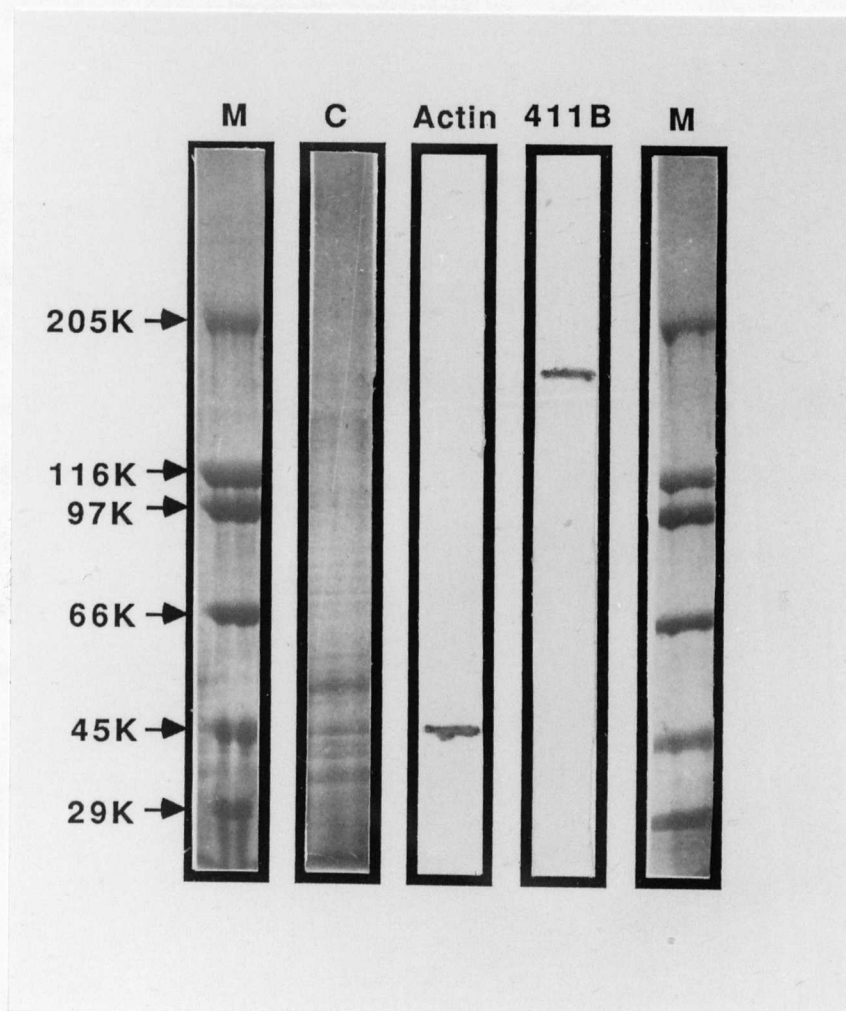


Figure 6iii) Western Blot of 411B and anti-actin. M = markers, C = Coomassie blue stain of rat hippocampus homogenate, Actin and 411B = hippocampus homogenate probed with anti-actin and 411B respectively.

6i) Subcellular distribution of R14 antigens in rat brain.

Subcellular fractions were prepared according to the method of Cotman and Taylor (1972). Whole rat brains were homogenised (10% w/v) in 0.32M sucrose and from the resulting homogenate were prepared a crude nuclear fraction, microsomes, cytosol and a mitochondrial fraction. SPMs were resuspended in 50mM Ca^{2+} and subjected to phase partitioning with 1-O-n-octyl-glucoside in order to isolate PSDs (Gurd, 1982).

The protein content of each fraction was determined using a modification of Bradford's method (Bradford, 1979). Each fraction was diluted to appropriate levels for ELISA as determined in pilot experiments (10-50 $\mu\text{g}/\text{ml}$). ELISAs were performed according to method described below. Absorbances read at 492nm were related to the protein concentration as determined using a modification of the Bradford method.

Results.

The relative distribution of the R14 antigens is presented in Figure 6iv). Compared to the parent homogenate there is an increase in the amount of the antigen in membraneous fractions. There is a slight elevation (+31%) in the whole membrane fraction with the antigen apparently more concentrated to synaptic membrane fractions. SPMs and PSDs contained 316% and 196% the amount of antigen respectively compared to that of the parent homogenate. This suggests that the antigen recognised by R14 is concentrated at, but not exclusive to, the synapse in rat brain.

R14 binding to rat s.c. fractions.

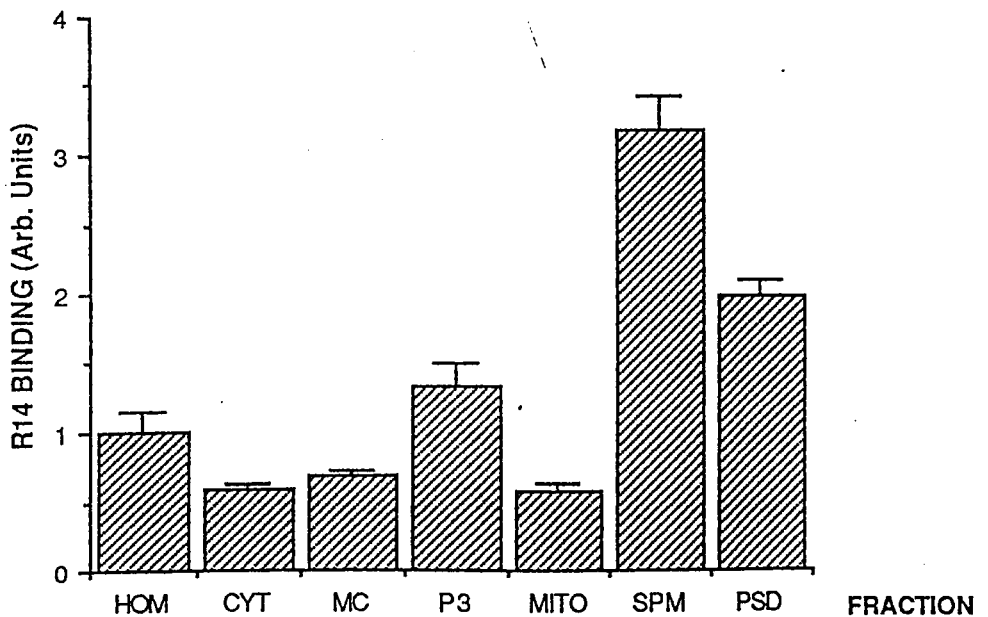


Figure 6iv) Distribution of R14 antigen in various subcellular fractions expressed as arbitrary absorbance units/[protein]. HOM = homogenate, CYT = cytosolic fraction, P3 = membranes, MITO = mitochondrial fraction, SPM = synaptic plasma membranes, PSD = postsynaptic density.

6ii) Time-course and Localisation of Posttetanic Changes in the Titre of Antibodies 411B and R14.

This experiment was aimed at establishing the time-course of the change in titre of 411B and to test for the possibility of the protein recognised by R14 being involved in plastic changes. To this end we assayed by ELISA lysates of hippocampal subfields dissected from the brains of rats which had undergone LTP. All samples were also assayed using an anti-actin polyclonal as a non-selective, control marker.

Electrophysiology.

Ten to 12 days prior to the experiment, 7-8 week old male Wistar rats were chronically implanted with teflon-coated, stainless steel, bipolar stimulating electrodes into the right perforant path (coordinates relative to the bregma: anterior-posterior, -6.9mm; lateral 4.1mm, vertical 4.1mm).

Tetanised animals

LTP was produced by applying four trains of 300 square wave pulses (200 Hz frequency, 300mA intensity) into the right perforant path. Each of the four trains was divided into 20 sets of 15 stimuli with an inter-stimulus interval of 5 sec. For each animal, the 4 trains were separated by intervals of 15 min. Polarisation effects at the tips of the stimulating electrodes were minimised by changing the polarity of the from pulse to pulse within each group. This procedure reliably produces LTP lasting for at least 72hr (e.g. Krug et al., 1984).

Passive Controls.

Passive controls were implanted with electrodes in the same way as the tetanised animals but were kept in their home cages throughout the experiment.

Preparation of Tissue Samples.

1, 5 or 24hrs following tetanisation, animals were killed, their brains removed and specific hippocampal loci (left and right CA1, CA3 and CA4/dentate area) were dissected out according to the method of Popov et al. (1973). Seven 0.5mm slices were cut from the dorsal hippocampus, from which hippocampal subregions CA1, CA3 and CA4/dentate area were removed (Lossner et al., 1975). Tissue samples from individual slice sections from each animal were pooled and immediately frozen on dry ice. The tissue samples were frozen prior to use whereupon they were thawed and homogenised in lysis buffer (5mM Tris-HCl, 50mM Ca^{2+} , pH 8.1, containing 1mM phenylmethylsulphonylfluoride (PMSF) as a protease inhibitor).

Immunoassays.

Antigen levels of the hippocampal subfields were determined using an enzyme-linked immunosorbent assay (ELISA) (Engvall & Perlmann, 1971) using the method described in Chapter 3 of this thesis. The protein content of each sample was determined using a modification of Bradford's method (Bradford, 1979) and diluted to an appropriate concentration for immunoassay (~30 $\mu\text{g}/\text{ml}$ for 411B and 10 $\mu\text{g}/\text{ml}$ for R14 and anti-actin). 50 μl /well of antibody was added at an appropriate dilution (1:500 for R14; 1:50 for 411B; 1:100 for anti-actin).

Results.

Results were calculated as arbitrary absorbance units/protein concentration for each region from each animal. The results are presented in tables 6i) - 6iii). For presentation purposes the mean from each region and at each timepoint was divided by the mean passive control value for that region and the time course of antigen levels for 411B, R14 and anti-actin, relative to a control value of 1, are presented in Figure 6v).

The antigens recognised by 411B and R14 displayed different patterns of immunoreactivity during LTP. 411B increased in immunoreactivity at 8hrs following tetanisation, specifically in the target, ipsilateral CA4/dentate area ($p < 0.001$). There was also an increase in other brain regions at 1hr, however this increase was far more generalised and the considerable spread of the data meant that no area approached a statistically significantly different level compared to controls. There was also a significant decrease in the left CA1 compared to passive controls ($p < 0.05$) although examination of the data reveals that this is probably a reflection of low levels of 411B binding in controls.

The data also confirms earlier results on the regional distribution of the 411B antigen (Bullock et al., 1990). There appears to be a disproportionately large amount of the antigen in homogenates taken from the dentate area of control animals as compared to the other two hippocampal subfields.

R14 displayed a far more general rise in immunoreactivity in all areas of the ipsi- and contralateral hippocampus examined. These differences reached a maximum and were significant at 24hrs after tetanisation; this elevation was highest in the target, ipsilateral CA4/dentate area (+72%; $p < 0.01$), and also reached significance in the ipsilateral CA1.

<u>411B levels.</u>					
	PC	Time post-tetaniisation			
		1hr	5hr	8hr	24hr
right CA1	1.550 (0.184)	2.700 (0.374)	1.226 (0.290)	1.550 (0.047)	1.636 (0.520)
right CA3	1.477 (0.134)	3.551 (0.348)	1.615 (0.357)	1.517 (0.253)	1.520 (0.377)
right CA4/ ad	2.139 (0.160)	2.913 (0.681)	1.930 (0.353)	2.867** (0.154)	1.808 (0.305)
left CA1	1.474 (0.325)	2.752 (0.517)	1.875 (0.439)	1.185 (0.096)	1.339 (0.271)
left CA3	1.339 (0.237)	2.229 (0.490)	1.946 (0.409)	1.339 (0.107)	1.865 (0.473)
left CA4 /ad	2.236 (0.313)	2.448 (0.398)	2.021 (0.424)	2.259 (0.251)	2.134 (0.366)

Table 6i) Mean binding levels for antibody 411B expressed as arbitrary absorbance units/[protein]. Figures are mean antigen levels in homogenate from hippocampal subfields CA1, CA3 and CA4/ area dentate taken from passive controls (PC) and 1hr, 5hrs, 8hrs and 24hrs following tetanisation of the right perforant path. Standard errors are presented in parentheses (**, $p < 0.01$ as determined by Mann Whitney U statistic; $N = 6 - 8$ in each group). (Values for the 8hr time point are taken from Bullock et al., 1990).

<u>R14 levels</u>	Time post-tetaniisation			
	PC	1hr	5hr	24hr
right CA1	2.078 (0.106)	2.240 (0.178)	2.465 (0.169)	2.932* (0.248)
right CA3	2.058 (0.307)	2.467 (0.217)	2.383 (0.248)	3.021 (0.552)
right CA4/ ad	1.934 (0.234)	2.468 (0.186)	2.574 (0.346)	3.326** (0.454)
left CA1	2.285 (0.182)	2.842 (0.122)	3.171 (0.481)	3.583* (0.710)
left CA3	2.410 (0.484)	2.598 (0.497)	3.080 (0.231)	3.735 (0.951)
left CA4 /ad	2.415 (0.228)	2.450 (0.475)	3.040 (0.390)	2.695 (0.457)

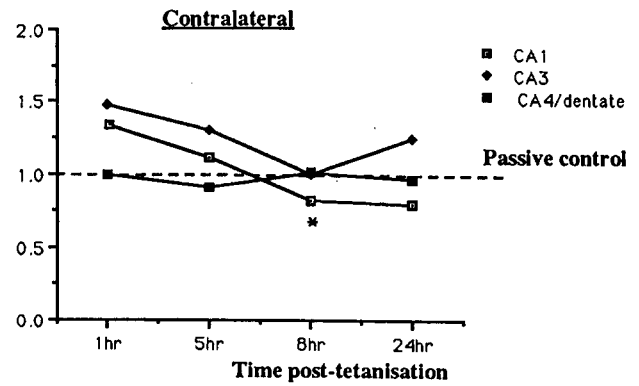
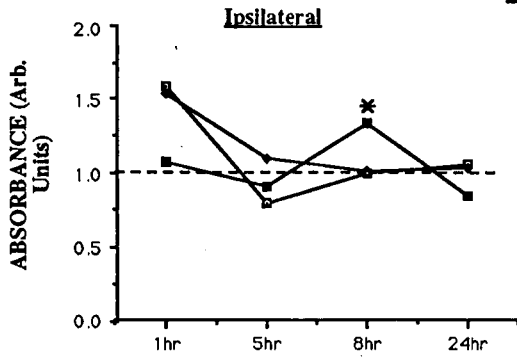
Table 6ii) Mean binding levels for antibody R14 expressed as arbitrary absorbance units/[protein]. Figures are mean antigen levels in homogenate from hippocampal subfields CA1, CA3 and CA4/ area dentate taken from passive controls (PC) and 1hr, 5hrs and 24hrs following tetanisation of the right perforant path. Standard errors are presented in parentheses (**, $p < 0.01$ as determined by Mann Whitney U statistic; $N = 6 - 8$ in each group).

<u>Actin levels.</u>				
	Time post-tetaniisation			
	PC	1hr	5hr	24hr
right CA1	1.862 (0.199)	2.067 (0.225)	2.191 (0.170)	1.933 (0.301)
right CA3	2.141 (0.325)	2.281 (0.236)	2.282 (0.212)	2.189 (0.375)
right CA4/ ad	1.822 (0.102)	1.665 (0.110)	2.089 (0.243)	1.700 (0.188)
left CA1	2.083 (0.209)	1.576 (0.205)	2.176 (0.117)	2.059 (0.343)
left CA3	2.163 (0.286)	2.477 (0.352)	2.338 (0.167)	2.164 (0.303)
left CA4 /ad	1.791 (0.058)	1.581 (0.292)	2.090 (0.210)	1.592 (0.241)

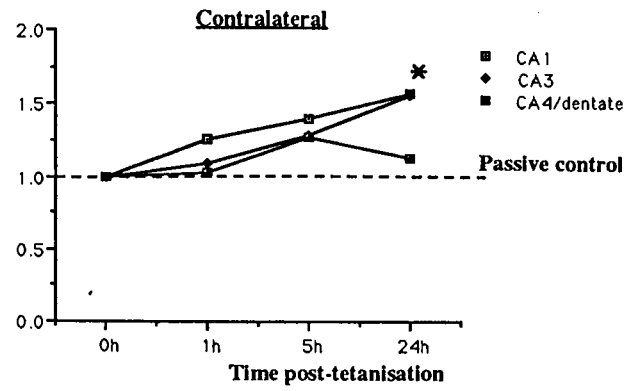
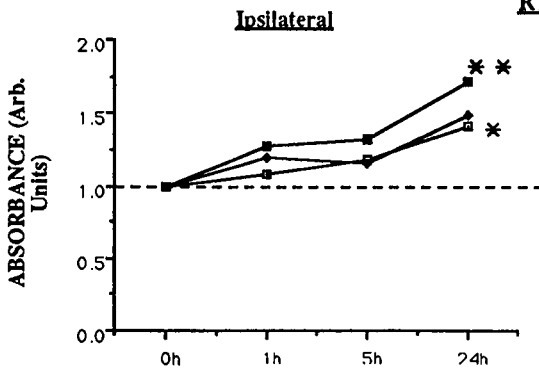
Table 6iii) Mean binding levels for anti-actin expressed as arbitrary absorbance units/[protein]. Figures are mean antigen levels in homogenate from hippocampal subfields CA1, CA3 and CA4/ area dentate taken from passive controls (PC) and 1hr, 5hrs and 24hrs following tetanisation of the right perforant path. Standard errors are presented in parentheses (N = 6 - 8 in each group).

Figure 6v) (Over) Mean binding levels of 411B, R14 and anti-actin expressed as arbitrary absorbance units/[protein]. Graphs show mean immunoreactivity in homogenates from hippocampal subfields CA1, CA3 and CA4/ area dentate taken 1hr, 5hrs, 8hrs and 24hrs following tetanisation of the right (ipsilateral) perforant path relative to a mean passive control value of 1. Standard errors are presented in parentheses (N = 6 - 8 in each group). *, $p < 0.05$; **, $p < 0.01$.

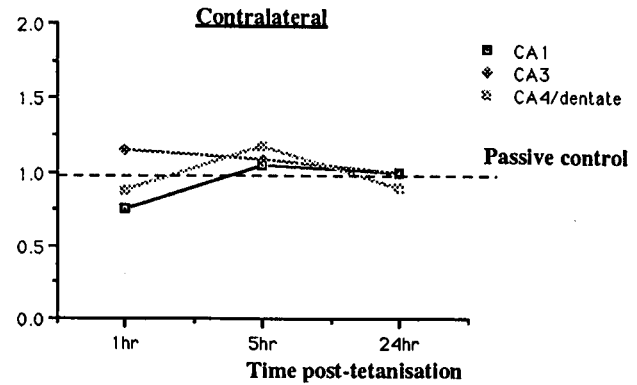
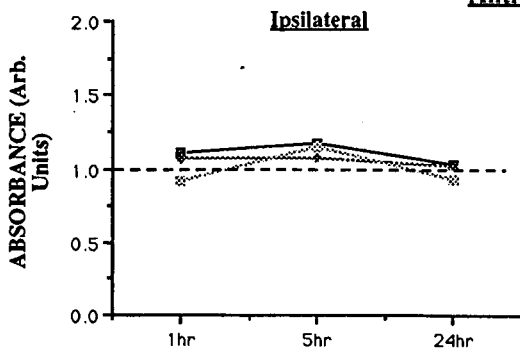
411B



R14



Anti-actin



(+41%; $p < 0.05$) and in the contralateral CA1 (+57%; $p < 0.05$). The levels in both the ipsi- and contralateral CA3 were also elevated but did not reach statistical significance ($p = 0.055$ and $p = 0.058$ respectively).

Anti-actin titres were not significantly different between tetanised animals and controls in any area at any time point.

6iii) Effect of Anisomycin on the Post-tetanic Titre of 411B.

The aim of this experiment was to determine if intraventricular infusion of the protein synthesis inhibitor, anisomycin had any effect on the 8hr change in the titre of 411B.

Methods.

The experimental procedure was similar to that described above with the exception that, at the time of the electrode implantation, animals were also implanted with an injection cannula into the ipsilateral ventricle.

The procedure for the induction of LTP was as above. Immediately after the tetanisation procedure experimental animals received an intraventricular infusion of 400 μ g of anisomycin in 5 μ l of saline. 8hrs following tetanisation the six hippocampal subfields were dissected out as described above. The regions were homogenised and ELISAs were performed on the homegenates in triplicate as described above. The resulting absorbances were divided by the protein content as determined using Bradford's method.

Results.

The results of the anisomycin experiment are presented in Table 6iv). There were no significant differences between passive controls injected with anisomycin and tetanised animals which received the same treatment.

	Condition	
	PC/ANI	TT/ANI
right CA1	2.752 (0.663)	2.149 (0.362)
right CA3	2.579 (0.445)	2.314 (0.258)
right CA4/ ad	2.027 (0.346)	2.197 (0.308)
left CA1	1.994 (0.259)	1.692 (0.554)
left CA3	2.616 (0.578)	1.651 (0.203)
left CA4/ ad	2.412 (0.350)	2.330 (0.474)

Table 6iv) Mean binding levels for antibody 411B in anisomycin-treated animals expressed as arbitrary absorbance units/[protein]. Figures are mean antigen levels in homogenate from hippocampal subfields CA1, CA3 and CA4/ area dentate taken from passive controls (PC/ANI) and 8hrs following tetanisation (TT/ANI). Standard errors are presented in parentheses (**, $p < 0.01$ as determined by Mann Whitney U statistic, $N = 6 - 8$ in each group).

DISCUSSION.

The differences in the results for the three antibodies used in this study indicate that they recognise proteins involved in very different cell biological processes. The specificity of the change in immunoreactivity of 411B implies that it may be involved in a particular post-tetanic change possibly reflecting an increase in the number or size of PSDs. In contrast, the antigen which R14 recognises shows an increase in all areas of the hippocampus suggesting that it is involved in a far less specific way in a generally increased function of the so-called "trisynaptic circuit" of the hippocampus.

The PSD is considered to be involved in many plastic changes within the nervous system. This could come about through its postulated role in constricting movement of membrane proteins, neurotransmitter receptors and ion channels which exist in the part of the membrane to which it is attached (Matus, 1978; Cotman and Kelly, 1980).

Siekewitz (1985) has proposed a model in which the PSD plays a cardinal role in determining enduring changes at the synapse. He argues that specific and general alterations in the PSD are primarily responsible for long-term changes in synaptic efficacy and that the PSD acts not only as an anchor for synaptic components, but also as a modulator of synaptic transmission. The theory is based on the contention that many of the reported biochemical changes which are concomitants of learning, development and electrophysiological stimulation reflect, or even predate, changes in the PSD structure. He also postulates that the morphology of the dendritic spine is determined by the PSD rather than by the dendritic cytoskeleton. It should be noted, however, that Siekevitz' model is highly speculative in places and, at best, based on correlative data. A hypothesis more specific to the LTP-associated changes in this study (and more data-driven) has been proposed by Desmond and Levy (1986a; 1986b) who examined several morphometric

measures of the PSD following tetanisation of the perforant path. They utilised two tetanisation regimes: one, the spaced tetanisation paradigm, consisted of four bouts of eight 8-pulse trains delivered once every 10 seconds with each bout being separated by 10min; the other, massed tetanisation, involved the delivery of one bout only of 24 high frequency stimulation trains delivered every 5 seconds.

Within the activated portion of the molecular layer of the dentate gyrus (i.e. the middle third), the total PSD surface volume of concave spine profiles increased significantly (+64% in the spaced paradigm and +35% in the massed) with a significant decrease (-25%) in the total PSD surface volume of non-concave spine profiles. The PSD surface area per synapse also increased markedly (+60% spaced, +126% massed) (Desmond and Levy, 1986a; 1986b).

The results obtained with antibody 411B are consistent with the data presented by Desmond and Levy even though in their study all measurements were taken within 60min of the cessation of tetanisation. If similar morphological changes account for the change in 411B immunoreactivity, the disparity in the timing of changes in their study and the 8hr change described in this thesis could reflect differences in the tetanisation procedure. It is also possible that changes observed by detailed layer-by-layer morphological analysis of the dentate gyrus would not be picked up by immunological assays of the dentate as a whole, indeed Desmond and Levy (1986a) found no significant changes between tetanised and control hippocampi in any measure of the PSD when the molecular layer of the dentate gyrus was taken as a whole. It is possible that such studies would detect even more changes in the PSD if the measures were performed on brains at longer time-points following LTP. Conversely, immunological assays of more localised subfields within the dentate may reveal changes in the titre of 411B at earlier post-tetanic time-points. It is possible, therefore, that the elevation in the immunoreactivity of 411B reflects an increase

in the functional surface of the PSD in which more of the glycoprotein containing the 411B epitope is inserted. This would allow for changes in the synaptic efficacy in the dentate gyrus as a concomitant of the electrophysiological changes observed with LTP.

The finding that the 8hr increase in the titre of 411B is abolished in the presence of anisomycin indicates that the molecule containing the epitope recognised by the antibody is newly synthesised in response to tetanisation rather than being subject to post-translational glycosylation. Alternatively the increase in the titre of the 411B antigen may be dependent on other proteins which are newly synthesised. This result is in accordance with previous experiments on the effect of anisomycin in an identical LTP procedure to that used in our experiment (Krug et al, 1984). Their study found that infusion of 400 μ g anisomycin resulted in an 80% decrease in protein synthesis (as measured by 3H-leucine incorporation), this resulted in a decay in LTP of the field EPSP and population spike 3-4hrs following tetanisation and that these measures were at control levels by 5-6hrs post-tetanisation.

The temporal specificity of the increase found in this study may reflect a phenomenon similar to that discussed in relation to chick and rat passive avoidance training. That is, the molecule recognised by 411B may play a role in the selection of synapses involved in a "stamping in" of something akin to a memory trace (or its LTP analogue) via the selection of synapses necessary for the maintenance of LTP. Observation of the data suggests that there is an early rise in the immunoreactivity of 411B 1hr following tetanisation in some hippocampal subfields (areas CA1 and CA3 of both the right and left hemisphere). The elevation in the titre, although not reaching significant levels, may reflect an early increase in the level of the antigen which is protein-synthesis independent and is involved in the establishment of an early phase of LTP. This then falls to near control levels before the specific increase in the target, dentate area. It should be remembered that, in the case of

chick passive avoidance, there is strong evidence for different brain loci being necessary for different phases of the memory trace (see preceding two chapters and thesis introduction). Recent studies have found a transient change in genomic expression in the dentate gyrus (Kaczmarek et al, 1989) suggesting that there is some signal which translates early synaptic events into long-term changes via activation of new genomic expression involving the expression of the c-fos protein. The results of this study are wholly consistent with such a model, whether protein synthesis is required simply to replenish the synaptic "stock" of the 411B glycoprotein is unclear.

It should also be stated that there is some cross-reactivity of 411B with glial cells (Bullock et al., 1988). Although there is very little research reported on a role for glia in synaptic plasticity, they are likely to play a role in phenomena such as learning and memory and LTP.

The picture for the changes observed with the R14 antiserum is less clear. In these experiments we found a generalised, post-tetanic increase in the immunoreactivity of all hippocampal subfields examined. This elevation reached significance 24hrs following tetanisation in the target, ipsilateral CA4/dentate area and, at the same time-point, in both the ipsi- and contralateral CA1 subfields. The perforant path does have projections to the contralateral dentate gyrus which is capable of producing weak LTP under certain conditions (Levy and Steward, 1983) although these projections comprise fewer than 10% of the total of those from the temporo-dentate pathway (Levy and Steward, 1983; White et al., 1988).

The dentate gyrus, CA1 and CA3 subfields of each hippocampal hemisphere are richly interconnected via the Shaffer collateral and mossy fibres, however, it is unclear why the increase in R14 titre is confined to area CA1 in the contralateral hemisphere. It is true that

all three areas of the ipsi- and contralateral hippocampus are elevated above control levels. This may reflect the involvement of the R14 rat antigen in some non-specific augmentation or neuromodulatory role within the hippocampus in response to tetanisation. Interestingly, no post-tetanic change in R14 titre was found in subfield CA3 of the hippocampus, an area which appears to exhibit NMDA receptor-independent LTP (Harris and Cotman, 1986).

The identities of the proteins containing the epitope recognised by 411B and R14 remain unknown at present. However, it has not escaped our attention that glycoproteins within the molecular weight range of the 411B antigen include members of the N-CAM family (e.g. Bock, 1989) and the synaptic glycoprotein, gp180 (Gurd, 1980; 1988), while the R14 antigen has a similar molecular weight and cellular distribution to that reported for fodrin (e.g. Groswald and Kelly, 1984). Furthermore, we cannot rule out the possibility of the diffuse, low molecular antigen recognised by R14 playing a part in the LTP-related changes reported. Which antigens are actually involved could be resolved by immunoblot analysis of control and tetanised subfields.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS.

In the introduction to this thesis, I briefly set out the goals which I hoped to achieve within the framework of this project. These included the disruption of memory formation by intracranial injection of anti-brain antibodies, the use of immunological techniques to identify changes in neural antigens following passive avoidance training in the chick and, finally, the mapping of changes in antigen levels in specific brain loci resulting from long-term potentiation in the rat. Since the specific remit of this thesis was to apply immunological techniques to these problems, results have, perhaps slightly more than in many other projects, necessarily relied on serendipity. Availability of antibody and, in the case of monoclonal antibodies, the chance of the relevant antigen being a functional protein with respect to synaptic plasticity, both determined the depth of analysis which could be undertaken. Indeed, I am aware that the main experimental chapters in this thesis (chapters 4, 5 and 6) deal with rather disparate results. However, the findings do contain common themes in addition to those simply of antibodies and synaptic plasticity. In this general discussion, I will attempt to bring together the threads of these results and place them in the context of reasonably coherent picture of synaptic plasticity. I will also set out what I feel would be a fruitful future research research programme based on these findings.

In chapter 3 the production and characterisation of an antibody raised by chronic immunisation with postsynaptic density protein was described. The antigens which antiserum R14 binds are synaptically-enriched and candidate proteins to which they may correspond were discussed.

The results described in chapter 4, established that intracranial injection of IgG from antiserum R14 resulted in amnesia for passive avoidance when administered 60min pre-training (but not 30min or 15min pre-training or 10min post-training) in chicks tested 24hrs

(but not 1hr or 3hrs) post-training.

One interpretation of these results is that the antiserum is effecting a second wave of glycoprotein synthesis which takes place in a region of the chick brain other than the IMHV. This second wave would take place between 3hrs and 24hrs post-training and indeed other data points to a time-window between 6hrs and 8hrs post-training (Doyle, 1990; Zamani and Rose, unpublished observations). It is possible that after such a time lapse antibodies to functional antigens have reached their target tissue and inhibit cell surface (possibly synaptic) antigens involved in consolidation of a memory trace. This may involve "capping" of synaptic antigens or the prevention of their movement within the plasma membrane. It is postulated that the antiserum-sensitive second wave may take place within the LPO of the chick brain.

In chapter 5 region-specific changes in the level of an α -tubulin epitope were identified. These changes, on the basis of earlier experiments (Mileusnic et al., 1980), could reflect an increase in absolute levels of tubulin. The changes in tubulin levels in birds trained on a methylantranilate-coated bead (M chicks) were significant when compared to water-trained (W) control animals with respect to the left IMHV and left LPO 1hr post-training, and the left IMHV and right LPO 6hrs and 24hrs post-training.

On the basis of evidence from lesion studies carried out in our laboratory, Rose has presented an information flow model of the regions involved in memory formation in the chick. According to this model the region responsible, or at least necessary, for acquisition of the task is the left IMHV. Sometime between 3hrs and 24hrs post-training the crucial region is the right IMHV, thereafter the right and then the left LPO become involved¹.

¹At the time of submission of this thesis the "information flow" hypothesis is being revised and replaced by a model in which the various regions of the chick forebrain code information regarding different stimulus components (size, colour, shape) of the training bead.

The data from my experiments with anti-tubulin indicate that an early stage of memory formation in the chick involves the left IMHV, as pointed to by lesion studies, and the left LPO, a region not indicated by lesion studies until later time-points. However, this substantiates results suggesting that the forebrain base is involved in a second consolidating wave of memory formation. The early elevation in brain tubulin in the left LPO does not of course mean that it is a necessary structure, merely that it is involved in some training-related process. The elevation of tubulin in the left IMHV persists through a 6hr time-point until at least 24hrs post-training. At these two later time points there is also an elevation in the level of tubulin in the right LPO. Thus the region-specific changes in tubulin levels overlap with those regions indicated by lesion studies but not exclusively so, suggesting that early events in the right IMHV and later ones in the left LPO are not dependent on tubulin or that changes in this protein are too subtle to be picked up using the antibody employed in this study.

Although lesions indicate that the left IMHV is necessary for acquisition only, there are several studies which point to biological changes within this region 24hrs post-training, including a dramatic increase in the number of dendritic spines in tissue of the left IMHV taken 24hrs post-training (Patel and Stewart, 1988; Patel et al., 1988). In chapter 5 a decrease in the levels of a MAP2 epitope, 24hrs post-training was reported. This may reflect a decrease in levels of the protein leading to a "molten" state of the dendritic cytoskeleton, thus allowing structural remodelling at spine synapses.

In chapter 6 there was a change in learning model from chick passive avoidance learning to long-term potentiation in the rat. In these experiments we found a change in the immunoreactivity of a postsynaptic density-specific monoclonal antibody (411B) 8hrs following LTP induction in the target dentate area. Again, the specific change in the putative glycoprotein recognised by 411B suggests that it may be involved in a second

wave of glycoprotein synthesis. We also found a general rise in the immunoreactivity of R14 which reached significance in three hippocampal areas (the target CA4/dentate and the ipsilateral and contralateral CA1) 24hrs following tetanisation.

The various timings of the changes measured in these experiments suggests that memory formation (for the purposes of this discussion I will include LTP within this phrase) results from a multiplicity of temporal and region changes within the brain and that the classical phraseology dividing memory into "formation" and "maintenance" is oversimplified.

The experiments described in this thesis have naturally generated some questions which could be the focus of further work. The results using R14 as an amnestic agent need to be clarified substantially. It would be interesting to establish which of the antigens recognised by R14 are functionally important in memory formation. One approach which may help in this endeavour would be to isolate the individual antibodies to each antigen using affinity blot purification (Olmstead, 1981). This method involves blotting heterogeneous antigens onto nitrocellulose paper (as described in chapter 3) and cutting the nitrocellulose into strips each containing one band. Having incubated the strips with antibody it is then possible to elute monospecific antibodies using an appropriate acidic eluant. The antibodies could then themselves be used to isolate larger quantities of pure antigen using affinity chromatography. Once isolated, the candidate antibodies could then be tested for brain specificity, synaptic enrichment, for the possibility of binding to glycoproteins and for potential amnestic action. Having established the methodology for using antibodies for amnestic effect in this thesis it should now be possible to use such antibodies for a more mechanistic description of synaptic events occurring as concomitants of memory formation in the chick. The R14 antibody which binds a 230kDa antigen and shows post-tetanisation changes would be a prime candidate for such treatment.

In the experiments reported in chapter 4 of this thesis a simple binary measure of peck or avoid was used. Perhaps future work on the amnestic effect of antibodies should use a measure such as peck latency to allow one to collect integral rather than merely categorical data.

It would also be interesting to see if intracranial administration of R14 or other antisera result in amnesia for tasks other than passive avoidance. These could include a passive avoidance task in which chicks discriminate between aversive and neutral beads of different colours thus establishing whether amnesic agents simply increase pecking by facilitating motor behaviour (e.g. Gibbs and Ng, 1980). Other tasks which have been used in the chick include a delayed sickness aversion task (Barber et al., 1990) in which presentation of a dry bead is followed, 30min later, by an intraperitoneal injection of the emetic lithium chloride and the bead is subsequently avoided. Tasks could also include a paradigm in which animals learn to discriminate between pebbles and egg white (used by Anokhin et al., 1991b).

The same techniques could also be employed making use of commercially available antisera and other antibodies made available to us from colleagues in the field of neurobiology. Antisera currently available include antibodies to the integrin family, N-CAMs, the ependymins PKC and its substrate, the B-50 phosphoprotein. Such antibodies could be used to assay for post-training changes in antigen levels and, in the case of the anti-glycoproteins, to establish if intracranial injections of the antibody result in amnesia. The anti-N-CAMs and anti-ependymins would be obvious candidates for such studies since it has already been established that they are amnestic in the rat and goldfish respectively (e.g. Doyle et al., 1990; Schmidt and Piront, 1985, see chapter 3).

Further insight into the functional role of amnestic anti-glycoprotein antibodies could be obtained by establishing whether amnesia can be "competed" with by injection of sugar precursors of the carbohydrate moiety of glycoproteins. For example, amnesia resulting from injection of 2-deoxygalactose has found to be reduced if fucose is injected along with the amnestic drug (Rose and Jork, 1987). If similar effects were found on, say, the R14 amnesia this would provide more insight into the mechanisms of amnesia, i.e. it would suggest that amnesia results from antibodies preventing the insertion of new carbohydrate into synaptic glycoproteins.

The use of such antibodies for behavioural pharmacology would have to be carefully controlled. I should emphasise that in this thesis I was particularly interested in defining the temporal parameters of amnesia using a rather heterogenous antiserum rather than in defining the exact biological process being disrupted. Using the antibodies described above as amnestic agents one would have to establish a far more precise susceptibility gradients and dose response curves. One way in which the amount of injected antibody could be optimised may be to inject antibody into the brain and then to use a variant of the method described in section 4vi) in which antibody actually in tissue was labelled using a peroxidase-conjugated anti-IgG. If, instead of probing crude homogenate, one could fractionate samples into membranes and cytosol and probe each separately for the amount of bound antibody, then the quantity of antiserum to be injected could be optimised. This would also reveal whether injected antibody saturates membrane sites before being found in soluble fractions.

The biological substrate affected by intracranial injection of R14 and, hopefully, other antibodies, should be more clearly defined. For example, does antibody-induced amnesia correlate with an inhibition of glycoprotein synthesis, neuronal spiking and/or an increase in spine density?

The results of section 4vi) of this thesis demonstrated that by 60min post-injection IgG does not diffuse from the IMHV into the PA or LPO. This technique could be developed to see if intact antibody survives the treatment involved in tissue slice staining to allow its further localisation. If, as preliminary results suggest, antibody does not diffuse throughout the brain within the first hour of injection then it may be possible to use antibodies for a kind of localised biochemical lesion which would have the great advantage of leaving connections to the relevant locus intact. One could therefore be more confident of the localised effect of, for example, unilateral injections of antibody as compared to other, smaller molecules used as amnesic agents. Another obvious question which could be addressed by this method would be to find out if injection of R14 directly into the LPO, timed so that antibody is binding at the time of the putative second wave of glycoprotein synthesis, results in amnesia.

With respect to the changes in immunoreactivity of the anti-tubulin antibody following passive avoidance training, there are several directions of investigation which could be followed. Firstly, which cellular functions are creating an extra demand for tubulin in the various loci examined? There is of course no reason to suppose that the different loci which show elevations in tubulin levels (or the same locus at different times) are using tubulin for the same processes. I have recently acquired a monoclonal antibody to the enzyme kinesin, a microtubule-bound motor which appears to play an ubiquitous role in anterograde axonal transport (e.g. J. Scholey et al., 1989). Having already established that the antibody cross-reacts with chick brain, it can now be used to determine if axonal transport, which may be reflected by an increase in kinesin levels, is one of the functions which correlates with the changes in tubulin levels in any of the regions already examined.

Further experiments should also be undertaken using the anti-tubulin antibody to determine the significance of the timing of loci-specific changes in tubulin levels. For example, if the

IMHV is lesioned at the time of training (an intervention which results in chicks being unable to learn the passive avoidance task), do changes in the tubulin levels in the forebrain base persist? Such an experiment would indicate whether later stages of "information flow" following passive avoidance training are dependent on earlier stages or if the later biochemical events are set in motion at the time of training.

The results found with respect to LTP could be followed up in a number of ways. One line of investigation would be to determine whether R14 or 411B antibodies are capable of inhibiting the establishment or maintenance of LTP. Probably the best way of achieving this would be through the application of IgG to hippocampal slices at various times relative to *in vitro* tetanisation. Other experiments could be aimed at establishing if the changes in R14 immunoreactivity are abolished in the presence of anisomycin or if the post-tetanic titres of the two antibodies are affected by inhibitors of glycoprotein synthesis. Using such methods with a variety of antibodies may lead to the discovery of a functional relationship between LTP in the rat and passive avoidance training in the chick.

As a final note I should mention some of the disadvantages, or at least shortcomings, of using antibody technology in the study of neuronal plasticity. Many neural proteins are notoriously non-antigenic, necessitating elaborate immunosuppression and enhancement techniques in order to obtain antisera. This problem may be reduced by using immunogens from, for example, chick brain (or invertebrates) to raise antibodies in rabbits and mice (as indicated by the finding that only one of the six R14 antigens is present in rat brain). It is also doubtful whether antibodies, because of their size and structure, could be used to disrupt intracellular processes *in vivo* as is possible using other inhibitors such as 2-deoxygalactose and inhibitors of protein synthesis. Lastly, and perhaps rather obviously, when using antibodies in the kind of studies undertaken in chapters 5 and 6 of this thesis (to map time- and locus specific changes following training), monospecificity is essential. We

learned this to our cost when trying to undertake experiments similar to those reported in chapter 4, using antibodies to spectrin and ependymin, only to find that the antisera were extremely heterogenous when blotted against the chick homogenates we had assayed. Despite these potential shortfalls, the power of using antibodies as selective and specific probes should not be underestimated.

This thesis has concentrated on developing applications for using the highly sensitive and selective methods of immunology in the study of synaptic plasticity. Future work should concentrate on using antibodies to both disrupt synaptic plasticity and to detect region- and time-specific changes in neural antigens. I hope that the work described in this thesis has done something towards setting the stage for such studies.

APPENDICES.

1. Protein Estimation.

The procedure for the estimation of protein content in samples was modified from the method of Bradford (1976) for use in 96-well plastic microtitre plates. The protein standards were prepared from commercial bovine serum albumin (BSA, Sigma) diluted from a stock solution of 1mg/ml with the protein concentration being based on the dry weight of the protein. Protein standards were prepared containing 100, 200, 300, 400, 500, 600, 800 and 1000µg/ml in distilled water.

10µl of each of these was added in triplicate to wells in microtitre test plates giving standards with absolute protein contents in the range 1µg - 10µg. 10µl aliquots of each sample were added to wells in triplicate at appropriate dilutions so that absorbances would fall into the linear range of the standard curve.

200µl of Bradford's dye (prepared by filtration of a solution 0.01% Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) orthophosphoric acid) was added to each of the standards and samples. The plate was gently agitated for approximately 5min and the plates read at 595nm in a commercial Titertek plate reader.

2. Preparation of tubulin.

Tubulin was obtained from taxol-polymerised microtubules prepared according to the following method (all steps at 4°C unless otherwise stated). Forebrain tissue (dissected from 100 day-old chicks) was homogenised by 4 X 30sec pulses in a commercial blender with 3ml/g of PMEG buffer (0.9% glycerol, 0.1M PIPES, 5mM EGTA, 2.5mM MgSO₄, 0.5mM EDTA, 1mM dithiothreitol, 0.1mg/ml soybean trypsin inhibitor, 10µg/ml leupeptin, aprotinin and pepstatin). The homogenate was centrifuged for 15min at 13,000rpm to remove cell debris. The supernatant was centrifuged for 45min at 41,000rpm, 20ml of P11 phosphocellulose was added to the resulting supernatant (177ml) and rocked for 1hr. The cellulose was centrifuged and taxol and GTP were added to the supernatant to a final concentration of 10µM and 1mM respectively. This mixture was incubated at 37°C for 30min to promote polymerisation of microtubules. The microtubules were pelleted and any associated proteins were "salt-stripped" by a 5min incubation at 4°C in 50ml PMEG containing 1M KCl, 10µM Taxol, 1mM GTP. The resulting microtubules were washed by pelleting X2 at 30,000rpm for 20min in PMEG buffer and stored frozen prior to use.

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